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(71) Applicant: DUKE UNIVERSITY [US/US]; 230 North Building, Research Drive, Box 90083, Durham, NC 27708–0083 (US).

(72) Inventors: PETERS, Kevin, G.; 3922 North Hampton Road, Durham, NC 27707 (US). LIN, Charles; 4 Blue Bird Court, Durham, NC 27713 (US). RAO, Prema, S.; 248 Seminole Drive, Chapel Hill, NC 27514 (US). DEWHIRST, Mark, W.; 212 Silvercreek Trail, Chapel Hill, NC 27514 (US).

(74) Agent: WILSON, Mary, J.; Nixon & Vanderhye P.C., 8th floor, 1100 North Glebe Road, Arlington, VA 22201–4714 (US).

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(54) Title: SOLUBLE TIE2 RECEPTOR

(57) Abstract

The present invention relates to a soluble Tie2 receptor and to the use thereof as an antiangiogenic agent.

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SOLUBLE TIE2 RECEPTOR

This application claims priority from Provisional Application No. 60/029,407, filed October 31, 1996, the entire contents of which are incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to a soluble receptor endothelium specific and to the use thereof as an antiangiogenic agent.

BACKGROUND

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is a fundamental process required for both normal embryonic development and the development of pathologic conditions such as cancer (Folkman, J. Natl. Cancer Inst. 82:4-6 (1990), Folkman et al, J. Biol. Chem. 267:10931-10934 (1992)). Tumor growth is an angiogenesis-dependent process that requires stimulation of new vessel growth (Viglietto et al, Oncogene 11:1569-1579 (1995), Kandel et al, Cell 66:1095-1104 (1991)). For example, in the absence of neovascularization, solid tumors will not grow beyond a few cubic millimeters (Folkman et al, J. Biol. Chem. 267:10931-10934 (1992)). Moreover, recent studies indicate that tumors with a luxuriant vasculature have a higher fraction of dividing cells and lower necrosis rates than tumors with a poorly developed vasculature (Folkman, J. Natl. Cancer Inst. 82:4-6 (1990), Lyng et al, Cancer Res. 52:584-592 (1992)). Recent clinical studies have shown a direct correlation between the density of tumor vessels and an adverse prognosis in patients with a variety of solid tumors including breast, colon, lung, kidney, bladder, and head and neck tumors (Albo et al, Ann. Plast. Surg. 32:588-594

(1994), Bosari et al, Human Pathology 23:755-761 (1992), Denijn et al, Clinical Aspects Melanoma Research 3:5-14 (1993), Guinebretiere et al, J. Natl. Cancer Inst. 86:635-636 (1994), Macchiarini et al, Lancet 340:145-146 (1992), Toi et al, Int. J. Cancer 55:371-374 (1993), Weidner et al, Am. J. Pathology 143:401-409 (1993), Weidner et al, J. Natl. Cancer Inst. 84:1875 (1992), Weidner et al, The New England J. Jed. 324:1-8 (1991)). Considering the importance of vascular growth in tumorigenesis, it seems certain that targeting the tumor endothelium will provide effective cancer therapy.

Tumor angiogenesis is most likely mediated by growth factors produced by tumor cells and/or by tumor infiltrating inflammatory cells such as macrophages or mast cells (Folkman et al, J. Biol. Chem. 267:10931-10934 (1992), Sunderkotter et al, J. Leukoc. Biol. 55:410-422 (1994)). These factors stimulate the proliferation, migration and morphogenesis of endothelial cells as a result of interaction with specific cell surface receptors. Although many factors likely contribute to vascular growth in tumors, VEGF (vascular endothelial growth factor) is currently the best candidate for an endogenous mediator of vascular growth (Connolly, J. Cell Biochem. 47:219-223 (1991), Ferrara et al, J. Cell. Biochem. 47:211-218 (1991)). A role for VEGF and VEGF receptors in tumor angiogenesis is supported by a growing number of reports demonstrating expression of VEGF and VEGF receptors in a number of different tumor types (Warren et al, J. Clin. Invest. 95:1789-1797 (1993), Plate et al, Can. Res. 53:5822-5827 (1993), Plate et al, Nature 359:845-848 (1992), Hatva et al, Am. J. Path. 146:368-378 (1995)). Furthermore, blocking the VEGF/VEGF receptor pathway inhibits the growth of a number of murine tumors and human tumor xenographs (Kim et al, Nature 362:841-844 (1993), Millauer et al, Can. Res. 56:1615-1620 (1996), Millauer et al, Nature 367:576-579 (1994)). Interestingly, however, a recent study has demonstrated that although many tumors are inhibited by

blockade of the VEGF/VEGF receptor pathway, others are unaffected suggesting that alternative pathways for vascular growth can drive tumor angiogenesis (Millauer et al, Can. Res. 56:1615-1620 (1996)).

Recently, a novel endothelium-specific receptor tyrosine kinase, Tie2 (a.k.a. Tek), was identified (Dumont et al, Oncogene 8:1293-1301 (1993), Iwama et al, Res. Comm. 195:301 (1993)). Tie2 was expressed predominantly in endothelial cell precursors (angioblasts) and in endothelial cells participating in angiogenesis (Schnurch et al, Development 119:957-968 (1993), Dumont et al, Dev. Dynamics 203:80-92 (1995)). Disruption of Tie2 function in transgenic mice resulted in embryonic lethality at day 8.5 due to defects in vascular development characterized by a reduction in endothelial cell number and a defect in the formation of microvessels (Sato et al, Nature 376:70-74 (1995), Dumont et al, Genes and Development 8:1897-1909 (1994)). Similar vascular defects occured following the disruption of a recently cloned Tie2 ligand (Suri et al, Cell 87:1171-1180 (1996), Davis et al, Cell 87:1161-1169 (1996)). These findings indicated that the Tie2 pathway was essential for the formation of the embryonic vasculature and suggested a role for Tie2 in pathologic angiogenesis including tumor neovascularization.

SUMMARY OF THE INVENTION

The present invention relates to a endothelium specific receptor inhibitor comprising an extracellular portion (domain) of an endothelium specific receptor, essentially free of transmembrane domain sequences, and a non-endothelium specific receptor sequence that is essentially non-immunogenic and/or that does not mediate the formation of dimers or oligomers.

Objects and advantages of the invention will be apparent from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B. Design and production of ExTek.6His. Schematic diagrams of the full-length Tie2 receptor and CSF-1 receptor, c-fms, together with their respective soluble extracellular domains fused at the carboxy-terminus to a 6 histidine tag (ExTek.6His and ExFms.6His) (Fig. 1A). Recombinant baculoviruses were constructed for the production of soluble, recombinant ExTek.6His and ExFms.6His by insect cells. ExTek.6His and ExFms.6His were purified from the conditioned media of baculovirus infected SF9 cells by one step Ni^{++}NTA agarose chromatography and analyzed by 7.5% SDS-PAGE followed by Coomassie staining (Fig. 1B). The expected 90 kd ExTek.6His protein (Lane 2) and 72 kd ExFms.6His protein (Lane 3) were purified to near homogeniety by this simple procedure. Lanes were loaded as follows: Lane 1, molecular mass markers; Lane 2, 0.5 μg of purified ExTek.6His protein; Lane 3, 1 μg of purified ExFms.6His; Lane 4, an equal volume of mock control solution purified from the supernatant of uninfected SF9 cells.

Figure 2. Inhibition of tumor growth in a cutaneous window chamber after a single administration of ExTek.6His protein. To determine whether inhibition of the Tie2 pathway for tumor angiogenesis could inhibit tumor growth, 200 μl of purified ExTek.6His protein (0.5 μg/μl) or an equal volume of control solution was placed directly in the window chamber at the time of tumor implantation. Ten days later, the animals were sacrificed and window chambers were removed for histochemical analysis. To compare the size of the ExTek.6His treated tumors vs. the control treated tumors, the tumor volume was calculated from the largest H & E stained tumor cross-section. Using this index, a more than

75% reduction in tumor growth was observed in ExTek.6His treated tumors vs. control treated tumors (n=9 pairs, $p<0.005$). Error bars indicate SE.

Figure 3. Inhibition of tumor vascularization by ExTek.6His protein. Tumor vessel length density was measured as an indicator of tumor vascularization from photomicrographs of window chambers bearing 10 day old ExTek.6His treated tumors or control treated tumors. A single administration of ExTek.6His protein resulted in an approximately 40% reduction in tumor vessel length density (n=9 pairs, $p<0.01$).

Figure 4. ExTek.6His protein did not adversely effect R3230AC tumor cell proliferation or viability. Cells were either maintained in the presence of ExTek.6His protein at 3 μM, which was approximately equivalent to the protein concentration used in the window chamber, or control solution for three days. Live cells were counted after suspension in PBS with 0.02% trypan blue on each following day. Each point represents the mean of 3 experiments.

Figures 5A and 5B. AdExTek expression. Expression of ExTek *in vitro* by AdExTek was tested by infection of cultured 293 cells with the virus at MOI=1 (Panel A). Three days after viral infection, a small amount of culture medium from AdExTek infected 293 cell was analyzed in a 8 % SDS-PAGE and then detected with a monoclonal antibody against Tie2. A strong ExTek band at molecular mass about 110kD was observed (Fig. 5A, lane 1). 0.1 μg of purified ExTek protein produced by recombinant Baculovirus was used as a positive control (Fig. 5A, lane 2). The molecular mass difference between AdExTek expressed recombinant ExTek with a strep tag and Baculovirus expressed recombinant ExTek with a 6-histidine tag was caused by different tag and

probably different glycosylation. Expression of ExTek protein *in vivo* using recombinant AdExTek was tested in Balb/c mouse (Fig. 5B). $5x10^8$ pfu AdExTek was injected iv through retro-obital sinus. The blood was collected by through the tail vein using a heparinized capillary tube and the plasma was recovered by brief centrifugation to remove the blood cells. ExTek concentration in plasma was determined by a simple ELISA assays. The ExTek concentration was calibrated by using purified recombinant ExTek protein produced in Baculoviral expression vector. High level of ExTek (more than 1 mg/ml) in blood was achieved two days after viral infection. The expression was transient, which slowed diminished to baseline in about 10 days.

Figures 6A and 6B. Inhibition of well established primary tumor growth by AdExTek. The effect of AdExTek on primary tumor growth was tested in two different kind of well established murine tumors. Murine mammary tumor 4T1 ($5x10^5$ cells per mouse) and melanoma F10.9 ($5x10^5$ cells per mouse) were implanted into the right flank of Balb/c and C57/BL mouse, respectively. After a palpable tumor was achieved (day 0), the mice were divided into two groups in each different tumor implanted animals. One group of mice were injected with control virus Adβ-gal directing the expression of β-galactosidase and the other group mice were injected with AdExTek virus iv through retro-obital sinus. The tumor size was measured afterward for 12 days. The ExTek expression was tested 2 days after viral injection. A significant inhibition on tumor growth was observed in AdExTek treated either 4T1 tumor group and F10.9 tumor group vs. control Adβ-gal treated animals. The inhibition was stronger during the first 6 days which correlated with high level expression of ExTek in the blood. The tumor growth inhibition decreased with the decrease of ExTek expression in

blood 6 days after viral injection. By the end of the experiments, there were 64% or 47% tumor growth inhibition for 4T1 tumor and F10.9 tumor, respectively.

Figures 7A-7D. AdExTek suppresses the tumor lung metastases growth. The inhibition of AdExTek on tumor lung metastasis was analyzed by measuring the lung weight and counting metastases on lung surface under a dissecting microscopy from both 4T1 tumor group (Figs. 7A and 7B) and F10.9 tumor group (Figs. 7C and 7D). As evidence of massive tumor metastasis, lung weight in control Adβ-gal treated 4T1 tumor group (Fig. 7A) or F10.9 tumor group (Fig. C) was more than twice of the AdExTek treated tumor groups or normal, uninjected mouse lung. There was no significant difference in lung weight between AdExTek treated group vs. normal mouse lung. By counting lung surface metastases, there were on average about 50 metastases found in control Adβ-gal treated both 4T1 tumor group (Fig. 7B) and F10.9 tumor group (Fig. 7D). In contrast, only a few lung surface metastases were visible in AdExTek treated either 4T1 tumor mice (Fig. 7B) or F10.9 tumor mice lung (Fig. 7D).

Figures 8A and 8B. Design and production of ExFlk.6His. (Fig. 8A) Schematic diagrams of the full-length VEGF receptor, flk-1, and the full length CSF-1 receptor, c-fms, compared to the recombinant soluble extracellular domains of flk-1 (ExFlk.6His) and c-fms (ExFms.6His) fused to a 6 histidine tag at the carboxy-terminus. (Fig. 8B) The soluble ExFlk.6His and ExFms.6His as well as a mock control solution were purified by one step Ni^{++}NTA agarose chromatography and analyzed by 7.5% SDS-PAGE followed by staining the proteins with Coomassie Brilliant Blue R-250. Lanes were loaded as follows: Lane 1, molecular mass markers; Lane 2, 1 μg purified ExFlk.6His protein; Lane

3, 1.5 μg purified ExFms.6His protein and Lane 4, an equal volume of mock control solution.

Figures 9A-9C. ExFlk.6His binds VEGF and inhibits VEGF mediated endothelial cell mitogenesis and migration in vitro. Fig. 9A. Saturation binding and Scatchard analysis of ExFlk.6His binding to VEGF. Purified ExFlk.6His was radiolabelled with $Na^{125}I$. Increasing amounts of ^{125}I labeled ExFlk.6His were then added to micro-wells pre-coated with VEGF (10ng/well) for 1 hour at room temperature. The wells were washed and radioactivity was detected by a r-counter. Nonspecific binding was determined by incubation of ^{125}I-labeled ExFlk.6His in the presence of 60-fold excess of unlabeled ExFlk.6His. All points are the average of duplicate determinations±SE. Scatchard analysis of these saturation binding data revealed a high affinity interaction (Kd=16nM). Fig. 9B. ExFlk.6His blocked VEGF -induced thymidine incorporation in endothelial cells. Human umbilical vein endothelial cells(HUVECs) starved for 24 hours in quiescence medium were stimulated with VEGF (10ng/ml), VEGF plus ExFlk.6His (2.5μg/ml) or VEGF plus ExFms.6His (2.5μg/ml). DNA synthesis was measured by pulse labeling for 3 hours with ^{3}H-thymidine 24 hours after stimulation. Single asterisk indicates significant difference from control ($p<0.0005$) and double asterisk indicates significant difference from VEGF-stimulated ($p<0.0005$). The experiment was performed in triplicates and repeated once with similar results. Error bars indicate standard error of mean. Fig. 9C. ExFlk.6His inhibited VEGF-induced endothelial cell migration. HUVECs were seeded in the upper chambers of Costar Transwell filter inserts (8μm pore size). The filter inserts with HUVECs were then placed in each well of a 24 well culture plate(lower chamber) containing 600μl of DMEM/BSA alone as control, DMEM/BSA plus human recombinant VEGF(10ng/ml), DMEM/BSA/VEGF plus

either ExFlk.6His or ExFms.6His and incubated at room temperature for 30 min. The migrated cells on the lower surface of the filter insert were counted after 4 hours incubation at 37°C. Single asterisk indicates significant difference from control ($p<0.0005$) and double asterisk indicates significant difference from VEGF-stimulated ($p<0.0005$). The experiment was performed in triplicate and repeated once with similar results. Error bars indicate standard error of all samples.

Figures 10A and 10B. ExFlk.6His formed heterodimers with endogenous, cell surface Flk-1 in the presence of VEGF. To test the possibility that ExFlk.6His could function as a "dominant negative" inhibitor, ^{125}I-ExFlk.6His was pre-incubated with either buffer, or an equimolar ratio of VEGF, VEGF plus excess ExFlk.6His, or FGF, and then allowed to bind to the cell surface of cultured endothelial cells(ECRF24). Bound ^{125}I-ExFlk.6His was measured by counting lysed cells using a g-counter (Fig. 10A). To demonstrate the formation of a high molecular mass heterodimeric complex, the above experiment was repeated except that a chemical cross-linker BS3 was added prior to cell lysis. After cross-linking, cell lysates were analyzed on 5% SDS-PAGE followed by autoradiography (Fig. 10B).

Figure 11. Mechanisms by which ExFlk.6His might inhibit VEGF receptor activation. Endogenous VEGF receptors are activated by ligand-mediated dimerization. Excess ExFlk.6His could function as competitive inhibitor, preventing receptor activation by simply competing for VEGF binding. Alternatively, ExFlk.6His could also undergo VEGF-mediated heterodimerization with the endogenous receptors on the cell surface and function as a "dominant-

negative" inhibitor. It is likely that both mechanisms contribute to the inhibitory action of ExFlk.6His on VEGF stimulated angiogenesis.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a soluble endothelium specific receptor inhibitor. The inhibitor is a fusion protein comprising an extracellular domain of an endothelium specific receptor fused to a non endothelium specific receptor sequence. The present invention further relates to a method of inhibiting angiogenesis using such a fusion protein. Endothelium specific receptors of particular relevance to the present invention include those that play a role in endothelial cell proliferation, migration and that are otherwise involved in vascular growth. Examples include Tie-1 (Partanen et al, Mol. Cell. Biol. 12:1698 (1992); Korhonen, Oncogene 9:395 (1994)) Tie-2 (Zeigler et al, Oncogene 8:663 (1992); Runting, Growth Fac. 9:99 (1993)), KDR (Terman, Bioch. Biophy. Res. Commun. 187:1579 (1992) (flk-1 (Matthews et al, Proc. Natl. Acad. Sci. 88:9026 (1991)), and flt-1 (deVries et al, Science 255:989 (1992)).

The fusion protein of the invention generally comprises an extracellular domain of an endothelium specific receptor, or ligand-binding portion thereof (see, for example, Davis-Smyth, EMBO J. 15:4919 (1996)), advantageously, Tie-2, and a non-endothelium specific receptor sequence. The extracellular domain is, preferably, essentially free of transmembrane domain sequences of the endothelium specific receptor. The non-endothelium specific receptor sequence of the fusion protein does not mediate formation of dimers or oligomers and/or is of low immunogenicity (eg is biologically inert, eg compared to Fc). The non endothelium specific receptor sequence is, preferably, not Fc. Preferred non

endothelium specific receptor sequences include polyhistidine (eg a 6-histidine tag) and a strep tag.

In the fusion protein of the invention, the non receptor sequence is, advantageously, C-terminal to the receptor sequence.

The present invention also relates to a nucleic acid (DNA or RNA) that encodes the above-described fusion protein. In particular, the present invention relates to a nucleic acid that encodes a fusion protein comprising the extracellular domain of Tie-2 or KDR (advantageously, human Tie-2 or KDR) fused N-terminal to a 6-His tag.

The present invention also relates to a recombinant molecule comprising the nucleic acid described above and to a host cell transformed therewith. Using standard methodologies, well known in the art, a recombinant molecule comprising a vector and a nucleic acid encoding the fusion protein of the invention can be constructed. Vectors suitable for use in the present invention include plasmid and viral vectors. Vectors into which a nucleic acid encoding the fusion protein can be cloned include any vector compatible with introduction into a selected host cell. Such vectors include any of a wide variety of commercially available plasmids, as well as baculoviruses, retroviruses, and adenoviruses. The nucleotide sequence of the invention can be present in the vector operably linked to regulatory elements, for example, a promoter. Suitable promoters include strong promoters, including inducible promoters. Specific examples include CMV, PGK and tumor cell specific and/or endothelial cell specific promoters such as Tie-1 or Tie-2 promoters.

As indicated above, the recombinant molecule of the invention can be constructed so as to be suitable for introduction a host cell. Suitable host cells include prokaryotic cells, such as bacteria, lower eukaryotic cells, such as yeast, and higher eukaryotic cells, such as mammalian cells, and insect cells. The

recombinant molecule of the invention can be introduced into appropriate host cells by one skilled in the art using a variety of known methods.

The present invention further relates to a method of producing the fusion protein as defined above. The method comprises culturing the above-described host cells under conditions such that the fusion protein encoding sequence is expressed and the fusion protein thereby produced.

The fusion proteins of the invention can be used as antigens to generate fusion protein specific antibodies, which are also within the scope of the invention. Methods of antibody generation are well known in the art. Both monoclonal and polyclonal antibodies are included, as are binding fragments thereof.

Compound Screens

The present invention also relates to methods of using the fusion proteins of the invention to screen compounds for their ability to bind to the extracellular domain of an endothelium specific receptor and thus to identify compounds that can serve, for example, as agonists or antagonists of angiogenesis. In a one screening assay, a fusion protein of the invention comprising a 6-His tag and bound via the tag to a solid support (eg to an affinity column) is contacted with a test compound and the ability of the compound to bind the fusion protein determined. Test compounds that bind are potential agonists or antagonists of angiogenesis. The contacting can be effected in the presence or absence of a natural ligand for the receptor the extracellular domain of which is present in the fusion protein. When contacting is effected in the presence of the ligand, competitive binding assay conditions can be used to determine whether the test compound enhances or inhibits binding of the ligand to the fusion protein. Compounds that inhibit binding of the natural ligand to the fusion protein can be

expected to be antagonists of angiogenesis and the reverse for compounds that enhance binding.

Screening procedures such as those described above are useful for identifying agents for their potential use in the treatment of various forms of pathologic angiogenesis, such as cancer, retinal neovascularization, arthritis and atherosclerosis as well as conditions characterized by reduced vascular density such as diabetes and hypertension. Agonists identified in accordance with the above screen can also be used to enhance wound healing.

Pharmaceutical Compositions

The present invention also relates to pharmaceutical compositions comprising, as active agent, the fusion proteins (and nucleic acids) of the invention. The invention also relates to compositions comprising, as active agent, compounds selected using the above-described screening protocols. Such compositions include the active agent in combination with a pharmaceutically acceptable carrier. The amount of active agent in the composition can vary with the agent, the patient and the effect sought. Likewise, the dosing regimen will vary depending on the composition and the disease/disorder to be treated.

Therapy:

The present invention also relates to methods of treating pathologic angiogenesis by modulating (e.g., inhibiting) binding of endothelial growth factors to endothelium specific receptors so as to preclude receptor activation (eg dimerization). This embodiment of the invention includes the use in gene therapy regimens of DNA sequences encoding the above-described fusion proteins. The encoding sequences can be present in a construct which, when introduced into target cells, results in expression of the DNA sequence and production of the

fusion protein. Target cells include endothelial cells, particularly endothelial cells present at the site of pathologic angiogenesis (eg, at a tumor site, retina, synovium, atherosclerotic plaque.) However, target cells can be distant from the site of pathologic angiogenesis so long as expression of the DNA results in circulating levels of the fusion protein sufficiently high to exert the effect sought (that is, enhancement or inhibition of angiogenesis).

For gene therapy to be practical, it is desirable to employ a DNA transfer method that: (1) directs the therapeutic sequence into specific target cell types, (2) is highly efficient in mediating uptake of the therapeutic polynucleotide into the target cell population, and (3) is suited for use *in vivo* for therapeutic application.

Delivery of the fusion protein encoding sequence can be effected using any of a variety of methodologies including transfection with a viral vector, fusion with a lipid, and cationic supported DNA introduction (see generally Verma et al, Nature 389:239 (1997)). Each of these techniques has advantages and disadvantages, so that the selection of which technique to use depends upon the particular situation and its demands.

Adenoviral vectors have been described for use in human gene therapy. Advantages of adenovirus vectors, particularly replication defective adenoviruses, include safety, the potential to carry large insert polynucleotide sequences, very high viral titres, ability to infect non-replicating cells, and suitability for infecting tissues *in situ*.

Alternatively, adenoassociated viruses, which integrate, can be used, as can other viral systems depending on the target site, or natural/engineered tissue tropism.

Gene transfer can be effected using replication-defective retroviral vectors harboring the therapeutic polynucleotide sequence as part of the retroviral genome

(Miller et al, Mol. Cel. Biol. 10:4239 (1990); Kolberg, J. NIH Res. 4:43 (1992); Cornetta et al, Hum. Gene. Ther. 2:215 (1991)). Advantages of retroviral vectors for gene therapy include the high efficiency of gene transfer into replicating cells, the precise integration of the transferred genes into cellular DNA, and the lack of further spread of the sequences after gene transduction.

Another gene transfer method suitable for use in humans is physical transfer of plasmid DNA in liposomes directly into cells *in situ*. Unlike viral vectors that must be propagated in cultured cells, plasmid DNA can be purified to homogeneity thereby reducing the potential for pathogenic contamination. Liposome-mediated DNA transfer has been described by various investigators (Wang and Huang, Biochem. Biphys. Res. Commun. 147:980 (1987); Wang and Huang, Biochemistry 28:9508 (1989); Litzinger and Huang, Biochem. Biophys. Acta 1113:201 (1992); Gao and Huang, Biochem. Biophys. Res. Commun. 179:280 (1991); Felgner, WO 91/17424; WO 91/16024). Liposomal compositions, however, may not possess the specificity necessary to deliver the exogenous DNA to all target cell types and non-physiological pH conditions may be necessary to effect fusion.

Immunoliposomes have also been described as carriers of exogenous polynucleotides (Wang and Huang, Proc. Natl. Acad. Sci. USA 84:7851 (1987); Trubetskoy et al, Biochem. Biophys. Acta 1131:311 (1992)). Immunoliposomes can be expected to have improved cell type specificity as compared to liposomes due to the inclusion of specific antibodies that bind to surface antigens on target cell types.

Behr et al (Proc. Natl. Acad. Sci. USA 86:6982 (1989)) reported using lipopolyamine as a reagent to mediate transfection, without the necessity of any additional phospholipid to form liposomes.

Low molecular weight polylysine ("PL") and other polycations are carriers that can be used to effect DNA-mediated transfection into cells. Zhou et al (Biochem. Biophys. Acta 1065:8 (1991)) have reported synthesis of a polylysine-phospholipid conjugate, a lipopolylysine comprising PL linked to N-glutarylphosphatidylethanolamine, which reportedly increases the transfection efficiency of DNA as compared to lipofectin, a commercially used transfection reagent.

Essentially, any suitable DNA delivery method can be used in the context of the present invention, including direct physical application of naked DNA comprising the expression construct/transgene to the target cell population.

The nucleic acid-containing compositions of the invention can be stored and administered in a sterile physiologically acceptable carrier, where the nucleic acid is dispersed in conjunction with any agents which aid in the introduction of the DNA into cells.

Various sterile solutions may be used for administration of the composition, including water, PBS, ethanol, lipids, etc. The concentration of the DNA will be sufficient to provide a therapeutic dose, which will depend on the efficiency of transport into the cells.

Actual delivery of the gene sequence, formulated as described above, can be carried out by a variety of techniques including *ex vivo* gene therapy or *in vivo* by infusion, IV, intra-articularly, or intra-ocularly.

The compositions containing the present fusion protein encoding sequences can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already affected by the particular disease/disorder, in an amount sufficient to cure or at least partially arrest the condition and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose" or "efficacious

dose". Amounts effective for this use will depend upon the severity of the condition, the general state of the patient, and the route of administration. It will be appreciated that combinations of fusion protein encoding sequences of the invention can be used, for example, sequences encoding the ExTek.6His and ExFlk.6His fusion proteins described in the Examples that follow.

In addition to the gene therapy approach described above, the fusion protein of the invention can be used directly to inhibit angiogenesis. Depending on the site of pathologic angiogenesis, the fusion protein can be administered, for example, systemically or locally, by injection, infusion, via a slow release device, or via a pump. While the amount administered and the dosing regimen will vary with the protein, the mode of administration, the patient and the effect sought, cancer treatment may be effected using for example, 50 μg-2mg doses, administered by IV infusion over 30 min to 1 hr, twice weekly, similarly arthritis treatment. Arthritis treatment can also be effected via local administration to the involved synovium. For retinal neovascularization, the fusion protein can be administered intraocularly via injection (eg at doses of 100 ng to 100 μg), scleral pumps can also be used. Atherosclerosis can be treated with systemic administration of the fusion protein or administration via a stent or balloon angioplasty. As above, the invention includes the administration of more than one fusion protein of the invention.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow.

EXAMPLE I

Experimental Details

Cell lines and antibodies

Recombinant baculovirus was generated and propagated in monolayer cultured SF9 cells maintained in Grace's Insect Medium Supplemented (Gibco/BRL) at 28°C. Protein expression was carried out in suspension cultured SF9 cells in Protein-Free Insect Medium (Pharmigen). The R3230AC rat adenocarcinoma cell line was maintained in DMEM plus 10% fetal bovine serum (Gibco/BRL) at 37°C with 5% CO_2. A mouse monoclonal antibody against the extracellular domain of human Tie2, which specifically recognized the Tie2 of several species such as mouse, rat and human was prepared (Wong et al, Circulation Res. 81:567 (1997)). The rat endothelial marker MRC-OX43 and biotinylated anti-mouse immunoglobulin was purchased from Harlan Bioproducts for Science and Dako Corporation, respectively. HRP conjugated Streptavidin and liquid DAB substrate kits were from Biogenex Corporation. Avidin/Biotin blocking and DAB enhancing solution were from Vector.

Construction of the ExTek.6His baculovirus vector and production of recombinant virus

Using two mouse Tek-specific cDNA primers (one : 5' GGATCCATGGACCTGATC 3', an Bam H I site was introduced in front of the start codon; the other: 3' CGTCTGGAGCCTAGCTA 5', a Cla I site was introduced at 5' end), the cDNA from nucleotide 124 to 2346 or amino acids 1-741 of mouse Tie2 (the entire extracellular domain except minus 3 amino acids at C-terminus) was amplified by RT-PCR from 9-12 day mouse embryo as previously described (Dumont et al, Oncogene 8:1293-1301 (1993)). The

resulting RT-PCR product was digested with Bam H I/Cla I and ligated to the same sites of the BSK Cla-/6His vector (BSK/ExTek.6His). BSK Cla-/6His is a modified BSK vector (Stratagene). The original Cla I site was deleted and a new Cla I site with an in frame 6-histidine tag followed by a stop codon was inserted into the BamH I and Xba I site. A 2.1 kb EcoR I/Not I fragment from BSK/ExTek.6His containing the mouse extracellular domain coding region followed by a six histidine tag was subcloned into the same sites of pVL 1393, a baculoviral expression vector (Pharmingen). The ExTek.6His transfer plasmid and Baculogold baculoviral DNA (Pharmingen) were co-transfected into SF9 cells for production of the recombinant baculovirus BvExTek.6His according to the manufacturer's instructions. Second passage virus was used to infect serum-free SF9 cells for ExTek.6His protein expression. The same approach was used to generate a recombinant baculovirus (BvExFms.6His) expressing the entire extracellular domain of human c-fms receptor fused to a 6-histidine tag at C terminus.

Purification of ExTek.6His protein

Spinner cultured SF9 serum free insect cells (1 liter) were infected with approximately 1 pfu/cell of second passage BvExTek.6His for 54 hours at 28°C. Cells were removed by centrifugation at 3000 rpm for 20 minutes at 4°C. The supernatant was dialyzed against 8 liters of PBS pH 8.0 for 48 hours with one change of buffer. The dialyzed supernatant was then incubated with 4 ml of Ni^{++}NTA resin (Qiagen). After 1 hour at room temperature, the resin-bound ExTek.6His protein was loaded onto a 10 ml column. The column was then washed with 200 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM Imidazole pH 8.0), and the protein was eluted with elution buffer (same was

wash buffer except containing 250 mM Imidazole) followed by a buffer change to PBS pH 7.2 by ultrafiltration (Centricon 10 from Amicon Co.). Control ExFms.6His was purified from SF-9 supernatant infected with BvExFms.6His virus and followed the same purification protocol. Mock control solution was generated from the supernatant of uninfected SF9 cells following the same purification procedure as for ExTek.6His protein. Aliquots of purified ExTek.6His and ExFms.6His protein and mock control solution were analyzed by SDS-PAGE on a 7.5% gel.

Tumor window chamber model

Tumors were grown in cutaneous window chambers in Fischer 344 rats (age 12-14 weeks, weight 140-160 grams, purchased from Charles River Labs, Raleigh, NC) as previously described (Papenfuss et al, Microvascu. Res. 18:311-318 (1979)). Briefly, two 1 cm diameter holes were dissected in opposing epithelial surfaces of the dorsal skin flap as it was retracted away from the posterior surface of the back. The underlying fascial was dissected away until two facial planes with associated vasculature remained. 100 µl of ExTek protein (0.5 µg/µl) or 100 µl of mock control solution was injected between the fascial planes. A 0.1 mm3 piece of R3230AC tumor from a donor rat was then placed onto the fascial plane and an additional 100 µl of ExTek.6His protein or control solution was added and the chambers were sealed with glass cover slips. The tissue within the chamber is approximately 200 microns thick and is semi-transparent. A pair of tumor window chambers were done at each time, one treated with ExTek.6His and the other with mock control solution. The tumor implants in each pair were tailored in similar size from the same larger piece of grossly viable tumor tissue. The areas of tumor implants were measured by using an image analysis software (JAVA, Jandel Co.). The baseline host vasculature, the blood flow and the

proximity of tumor tissue to vessels were scored. Tumor growth and neovascularization was photographed using a dissecting microscope (Zeiss, Stemi SV6) on days 5, 7 and 10 and window chambers were harvested for H & E staining on day 10. For immunohistochemistry, a window chamber bearing a 5 day old untreated R3230AC tumor was freshly frozen in OCT.

Measurement of tumor volume and tumor vascular length density

To obtain the tumor size, H & E stained sections representing the largest cross-sectional area of each tumor were photographed and the thickness (t) and the diameter (d) of tumors were measured from the photographs. Tumor volumes, which were assumed to approximate a flat cylinder in shape, were calculated using the formula:

$$\text{Tumor volume } (mm^3) = 3.14t(d/2)^2$$

Tumor vascular length density as an indicator of tumor vasculature was measured from photographs of 10 day old tumors within the window chamber using a previously described method (Dewhirst et al, Radiat. Res. 130:345-354 (1992)). 3-5 areas inside the tumor were randomly selected for measurement. The vascular length density in mm/mm^3 was calculated using the formula:

$$\text{Length density} = N/(4gdt)$$

where N is the average number of intersections between vessels and grid per sheet; g is number of blocks in grid (=54); d is length of one grid square calibrated by a micrometer image at the same magnification (=0.1333 mm); and t is measured depth of field through which microvessels could be discerned (=0.2 mm).

Rat corneal micropocket assay

In vivo angiogenic activity of ExTek.6His was tested in the avascular cornea of F344 female rat (Harlan Labortories, Madison, WI) as described (Papenfuss et al, Microvascu. Res. 18:311-318 (1979)). Briefly, each sample was combined with sterile Hydron casting solution (Interferon Sciences, New Brunswick, NJ), and the solution was pipetted onto the surface of 1.5 mm diameter Teflon rods (Dupont Co. Wilmington, DE). The pellets were air-dried in a laminar hood for 1 hour and refrigerated overnight. The following day, pellets were rehydrated with a drop of PBS buffer and then placed in a surgically created pocket within the cornea stroma, 1.5 mm from the limbus. Corneas were observed every other day until day 5 or 7 when the animals were anesthetized and perfused with lactated ringers solution followed by colloid carbon solution to enumerate the vessels. Responses were scored as positive when vigorous and sustained directional ingrowth of capillary sprouts and hairpin loops toward the implant were detected. Negative responses were recorded when no growth was detected or when there was only an occasional sprout or hairpin loop with no evidence of sustained growth. Positive controls consisted of Hydron pellets containing 25 ng/5 μl pellet. Negative controls consisted of sham implants and Hydron pellets containing media alone. Media was incorporated into pellets at a concentration of 1 μg of total protein per cornea. Representative corneas were examined histologically and except for occasional neutrophils found in the limbus of both control and test corneas, nonspecific inflammation was not a contributing factor in any of the corneal responses.

In vitro cell proliferation

R3230AC tumor cells were seeded at $4x10^4$/well into 12 well plates and maintained in the presence of purified ExTek.6His protein (3 μM) or control

solution. Cell morphology was monitored every day by light microscope. Live cells were trypsinized, suspended in PBS containing 0.02% trypan blue (Gibco/BRL) and counted with a hemocytometer each following day for three days.

Histochemistry and anti-Tie2 immunohistochemistry

Tumor windows to be processed for H & E staining were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. Samples for immunohistochemistry were freshly frozen in liquid nitrogen and embedded in OCT. For immunohistochemistry, serial sections of 10 microns were cut, fixed in ice cold acetone for 10 minutes, and blocked with 0.03% H_2O_2, 1% horse serum and avidin/biotin blocking reagents (Vector). After blocking, sections were incubated with a monoclonal anti-Tie2 antibody or an antibody specific for rat endothelium (MRC-OX43) in a humidified chamber for 50 minutes at room temperature. With PBS washes between all steps, a biotinylated linker antibody (Dako) was applied for 30 minutes, followed by another 30 minute incubation with streptavidin linked HRP. Peroxidate activity was localized with DAB (diaminobenzidine) and enhanced by DAB enhancing solution (Vector). Sections were then weakly counter stained with hematoxylin.

Statistics

Results are reported as mean±SE for tumor volume and tumor vascular length density for each group. A two-tailed Student's t test was used to analyze statistical differences between control treated group and ExTek treated group. Differences were considered statistically significant at $p<0.05$.

Results

Design and Production of ExTek.6His fusion protein

To produce large amounts of properly processed ExTek protein and a control protein, ExFms (extracellular domain of the CSF-1 receptor), recombinant baculovirus vectors directing the expression of ExTek or ExFms protein fused to a 6-histidine tag were constructed (Figure 1A). ExTek.6His and ExFms.6His were purified from the supernatant of baculovirus infected SF9 cells by one-step Ni^{++}NTA resin chromatography, yielding a single major band of the expected molecular mass for each protein (Figure 1B, Lanes 2 and 3, respectively). The identity of the ExTek protein was further confirmed by Western blot by using a monoclonal anti-Tie2 antibody. A mock control solution was purified from uninfected SF9 cell supernant using the same protocol (Figure 1B, Lane 4).

ExTek.6His blocks angiogenesis stimulated by tumor cell conditioned media

First the ability of ExTek.6His to block angiogenesis in rat corneas was tested (Polverini et al, Methods Enzymol. 198:440-450 (1991)). Hydron pellets containing either fresh media (5 µl), ExTek.6His (100 ng) or ExFms.6His (100 ng) alone failed to stimluate an angiogenic response in rat cornea 5 to 7 days after implantation (Table 1). However, a strong angiogenic response was seen with pellets containing R3230AC tumor cell conditioned media (1 µg total protein). This angiogenic response was completely blocked by the addition of 100 ng of ExTek.6His into the pellets containing tumor cell conditioned media. Addition of control protein ExFms.6His (100 ng) to pellets containing tumor cell conditioned media did not block the angiogenic response. These results demonstrated that ExTek.6His specifically and potently inhibited corneal angiogenesis induced by tumor conditioned media *in vivo*.

TABLE 1. Inhibitory Effect of ExTek.6His on Angiogenic Response Induced by Tumor Conditioned Media

Samples	Portion of Corneal Angiogenic Response(%)	
	Negative	Strong Positive
Fresh Media	3/3 (100%)*	
ExTek.6His	2/2 (100%)	
ExFms.6His	4/4 (100%)	
Conditioned Medium (CM)		3/3 (100%)
CM + ExTek.6His	4/4 (100%)	
CM + ExFms.6His		3/3 (100%)

*n/m (x):m is the number of rats used in each experiment; n is the number of rats with the indicated response; x is the percentage of rats with the indicated response.

Tie2 is expressed in tumor vessels at the onset of tumor angiogenesis

To address the role of Tie2 in tumor angiogenesis and tumor growth, purified ExTek.6His protein was used as an inhibitor in a rat cutaneous window chamber bearing a R3230AC mammary tumor. Previous work has demonstrated that small fragments of tumor ($0.1mm^3$) placed in a window chamber become vascularized and undergo rapid growth within 10-14 days (Dewhirst et al, Radiat. Res. 130:345-354 (1992)). Typically, vascularization of tumors in the window chamber is first detected at about 5 days after implantation and is followed by a rapid growth phase of the tumor. Consistent with a role for Tie2 in vascularization of the R3230AC mammary tumor, immunohistochemical studies

demonstrated expression of Tie2 in vessels surrounding and penetrating the tumor implant at 5 days after implantation.

ExTek inhibits tumor growth in cutaneous "window chambers"

To determine the functional significance of Tie2 expression during tumor angiogenesis, a single dose of purified ExTek.6His protein (100 μg) was administered directly into the window chamber at the time of tumor implantation. After 10 days, gross inspection of live tumors in the tumor window and examination of histologic section, demonstrated that tumors (n=9 pairs) treated with ExTek.6His protein were smaller, contained more connective tissue, and had a less well developed vasculature with no clear hypervascular boundary as compared to tumors in the control group. Comparison of tumor size by estimation of tumor volume confirmed that the ExTek.6His treated tumors were on average about 75% smaller than the mock control treated tumors (Figure 2). No inhibition of tumor growth was observed when ExFms.6His was used and an alternative form of ExTek possessing a"strep-tag" gave a similar growth inhibition.

ExTek.6His inhibits tumor vascularization

The above results indicated that inhibition of tumor angiogenesis by ExTek limited tumor growth. To support this notion, tumor vascular length density was measured from photomicrographs of tumor window chambers bearing ExTek.6His treated or mock control treated tumors 10 days after implantation. Consistent with inhibition of tumor angiogenesis, there was an approximately 40% reduction in tumor vessel length density in ExTek.6His treated tumors vs. mock control treated tumors (n=9 pairs, $p<0.01$, Figure 3). Considering the documented strong link between tumor growth and tumor angiogenesis, the ability

to measure any decrement in tumor vascular density indicated that the primary action of the ExTek.6His protein was to inhibit tumor neovascularization.

ExTek.6His does not directly affect tumor cell proliferation or viability

To further confirm that the primary effect of ExTek6His was on the tumor vasculature, the cytotoxicity of the ExTek.6His protein on cultured R3230AC cells was assayed. When cells were cultured in the presence of 3 μM of ExTek.6His protein, no difference in tumor cell proliferation or viability was observed compared to control (Figure 4). This result indicates that inhibition of tumor growth by ExTek *in vivo* was not mediated by direct tumor toxicity.

EXAMPLE II

Experimental Details

Cell lines and antibodies

Recombinant adenovirus was generated and propagated in monolayer cultured 293 cells maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco/BRL) at 37°C with 5% CO_2. The murine mammary carcinoma cell line 4T1 and murine melanoma cell line F10.9 were maintained in DMEM plus 10% fetal bovine serum at 37°C with 5% CO_2. Mouse monoclonal anti-Tie2 antibodies were produced (Wong et al, Circulation Res. 81:567 (1997)).

Construction of recombinant AdExTek

A novel adenoviral vector system, in which partial E1 region and all the E3 region were deleted, derived from the in340 strain of adenovirus type 5 (ad5) was used in construction of AdPac β-gal (Channon et al, Cardiovascular Res. 32:962-972 (1996)). The β-gal gene was inserted in E1 region. The resulting

viral vector, AdPac β-gal, served as a control virus and was used to generate AdExTek by replacing the β-galactosidase gene with one containing ExTek cDNA. The ExTek cDNA containing the mouse extracellular domain coding region fused to a strep-tag at C terminal was subcloned into BamH I and Nhe I site of the transfer plasmid pGEM CMV/BGH poly(A). The plasmid pGEM CMV ExTek/BGH poly (A) was then digested with Xba I and Bst B1, and the 3.6 kb plasmid fragment consistent with the early CMV promotor and enhancer, ExTek and BGH was directly ligated to the Xba I site of parent virus Ad.Pac β-gal DNA. The ligation mixture was purified by phenol-chloroform extraction and ethanol precipitation, then transfected into 293 cells using the calcium phosphate method. After observation of a cytopathathic effect (7-10 days), the cells were lysed by multiple freeze/thaw cycles, and the recombinant virus was isolated by plaque assay on 293 cells.

Propagation and purification of recombinant adenoviruses.

Plaque purified AdExTek or Ad.Pac β-gal virus was used to generate high titer virus stock by infecting forty 150 mm plates of confluent 293 cells at a multiplicity of infection of 2 in DMEM plus 2% FBS. The viruses were purified from the infected cell lysates as described previously (Channon et al, Cardiovascular Res. 32:962-972 (1996)). The virus was stored in virus storage buffer (VSB; 20 mM Tris pH 7.4, 150 mM NaCl, 5 mM KCL, 1 mM $MgCl_2$) plus 1 mg/ml mouse albumin (Sigma) and 10% glycerol and immediately frozen in aliquots at -80°C. Titers of viral stocks were determined by plaque assay on 293 cells using standard techniques (Channon et al, Cardiovascular Res. 32:962-972 (1996)).

Determination of ExTek concentration in blood.

ExTek concentration in blood was determined by a simple ELISA assay. Briefly, AdExTek adenovirus ($5x10^8$ pfu) was injected into Balb/c mouse circulation through retro-obital sinus. Two days later, small amount of blood was collected in a heparinized microcapillary tube from tail vein. Mouse plasma was recovered after brief centrifugation to remove cells. Serial dilutions (in PBS) of the plasma were incubated in micro wells overnight at 40°C. The coated wells were blocked with 5 % milk in TBST (10mM of Tris Cl, pH 8.0, 150mM of NaCl, 0.05% of Tween 20) for 30 minute. With 3 washes of TBST in between each step, a biotinylated mouse anti Tie2 monoclonal antibody (Ab33) diluted in TBST (0.5 μg/ml) was incubated for 1 hour followed by incubation with 1:2000 diluted strep-avidin alkaline phosphatase conjugate (Gibco/BRL) in TBST for 30 minutes. The phosphatase activity was determined by addition of Sigma @104 phosphatase substrate dissolved in diethanolamine and 0.5 mM $MgCl_2$, pH 9.8, and absorbency at 405nm was measured in V_{max} Kinetic Microplate Reader (Molecular Devices). The ExTek concentration in blood was calibrated using purified recombinant ExTek in the ELISA assay. All experiments were carried out at room temperature except as otherwise indicated.

Primary tumor growth.

To determine the inhibitory effect of AdExTek on well established primary tumor growth, a murine mammary tumor cell line 4T1 or a murine melanoma cell line F10.9 was implanted into the flank of female Balb/c mouse or C57/BL, respectively ($5X10^5$cells/mouse in 50μl PBS). Following the development of an easily palpable tumor (7-10 days), either AdExTek virus or a control Adβ-gal virus ($5x10^8$ pfu/mouse in 100 μl PBS) was injected into

circulation through retro-obital sinus. The tumor size was then measured using a caliper every other day for the following 12 days. The tumor volume was calculated using the following formula: Tumor volume = 1/2(width)2 x Length. Student t-test was used to calculate standard deviation. The ExTek concentration in plasma was checked two days after viral infection.

Tumor metastasis.

$5X10^5$ of either tumor cell lines 4T1 or F10.9 was freshly mixed with $5X10^8$ of either AdExTek or equal amount of control Adβ-gal viruses and co-injected into female Balb/c or C57/BL mice, respectively, through retro-obital sinus. About twenty to twenty five days after injection, the animals were sacrificed and the lungs were removed, weighed and fixed in Bouin's solution. Surface metastases were then counted under a dissecting microscope.

Histochemistry

Mice lungs to be processed for H & E staining were fixed in Bouin's solution for a few days, decolorized in 70% ethanol several times before being embedded in paraffin. Serial sections of 8 microns were cut. The sections were stained with H&E for histoligic examination.

Results

Construction of a recombinant adenovirus for gene transfer of ExTek

To test the efficacy of systemic delivery of ExTek to block growth and tumor metastasis, a recombinant adenovirus (AdExTek) was constructed, which was based on a E1 and E3 double deleted Ad5 vector. ExTek cDNA was inserted in E1 region driven by the CMV promotor.

The efficacy of AdExTek gene transfer was initially evaluated in cultured 293 cells. The 293 cells at 75% confluence were infected with AdExTek at MOI=1. The infected cell media was harvested three days after viral infection, and analyzed by 8% SDS-PAGE. The separated protein bands were transferred onto nitrocellulose membrane and then blotted with anti-Tie2 Ab33. Purified recombinant ExTek protein expressed in insect cells using a viral vector as a positive control (Lin et al, J. Clin. Invest. 100:2072-2078 (1997)). The result demonstrated that a high level of ExTek protein was produced and secreted into the medium after infection of cells with the AdExTek virus (Fig 5A). The molecular mass difference between baculovirus expressed ExTek and AdExTek virus produced ExTek may be due to the different epitope tag and different glycosylation.

Efficacy of ExTek expression in vivo

To evaluate the efficiency of AdExTek gene transfer *in vivo*, a simple ELISA assay was used to determine the ExTek concentration in plasma. AdExTek was administered to Balb/c mice in intravenous injection into the retro-orbital sinus. The plasma was collected every other day after viral injection. Serial dilutions of the plasma were then incubated in ELISA plates. The ExTek content was determined using biotinylated anti-Tie2 antibody (Ab33). The concentration of ExTek was calibrated using purified recombinant ExTek (Lin et al, J. Clin. Invest. 100:2072-2078 (1997)). High level expression could be detected in the plasma (1-2 mg/ml) two days after viral injection. The high level expression was transient with levels falling to baseline within 12 days (Fig 5B). The mice with high levels of circulating ExTek showed no apparent ill effects, no weight loss and no behavioral abnormalities.

AdExTek inhibits the growth rate of two well established primary murine tumors

To determine whether administration of AdExTek could inhibit the growth rate of well established primary tumors, two murine tumor cell lines, a mammary adenocarcinoma (4T1) and a melanoma (F10.9.). Tumor cells ($5x10^5$ in 50 µl of PBS) were implanted into the flank of female Balb/c mice (for 4T1) or female C57/BL (for F10.9). Following the development of an easily palpable tumor (7-10 days, approximate over 4 mm diameter), either $5x10^8$pfu of AdExTek or a control virus directing the expression of β-galactosidase was injected IV through retro-obital sinus. The tumor size was then measured every other day for 12 days. In this experiment, transient expression of ExTek after administration of AdExTek significantly inhibited the growth rate of both tumors *in vivo*, compared to control virus injected animals. At day 12 after viral injection, AdExTek treated tumors were much smaller compared to tumors in control virus treated animals; tumor volume was 64% or 47% smaller for mammary tumor (4T1) or melanoma (F10.9), respectively (Figs. 6A and 6B). These results demonstrated that blocking the Tie2 pathway by systemic delivery of ExTek using adenoviral gene transfer could inhibit solid tumor growth even though both tumors were well established before the treatment.

AdExTek suppress tumor metastasis

To determine whether blocking Tie2 activation could suppress the growth of tumor metastases, two highly metastatic cell lines 4T1 and F10.9 ($5x10^5$) were mixed with $5x10^8$ pfu of either the AdExTek virus or the control AdPac β-gal virus and then co-injected into the circulation through retro-obital sinus. About 20 days after injection, 3 mice injected with control virus died from massive lung metastases but all of the mice that received AdExTek were alive. The remaining

animals were sacrificed and the lungs were removed, weighed and fixed in Bouin's solution. Lungs were grossly examined under dissecting microscopy. As evidence of massive metastasis, numerous lung surface metastases were formed in control virus treated both 4T1 tumor and F10.9 tumor. In contrast, there were none or very few visible lung surface metastases could be seen in AdExTek treated both 4T1 tumor and F10.9 tumor. Comparison of tumor metastasis by counting the lung surface metastases (Figs. 7B and 7D) and by measuring the lung weight (Figs. 7 A and 7C) confirmed that the lungs of remaining control treated both tumor group mice weighed more than twice as much as those of normal, uninjected mice (0.34g vs 0.16 g for 4T1 group mice; 0.53g vs 0.17g for F10.9 group mice) and had numerous gross visible lung metastases (on average , 52 metastases in 4T1 group mice; 50 metastases in F10.9 tumor group). In contrast, the lungs of the AdExTek treated mice weighted the same as or very close to the lungs of normal, uninjected mice and had no or very few visible surface metastasis (on average , 2.5 metastases in 4T1 group mice; 8.2 metastasesin F10.9 tumor group).

AdExTek suppress tumor lung metastases from growing

To explore the mechanism of inhibition of tumor metastasis by AdExTek, the animal lungs were harvested, fixed in Bouin's and embedded in paraffin and sectioned for histochemistry and immunohistochemistry examination. H&E stained lung sections revealed that large well vascularized pulmonary metastases in both tumors 4T1 and F10.9 in AdPac β-gal control virus treated lungs. In contrast, only micro-metastases could be seen in both tumors 4T1 and F10.9 (panel D) in AdExTek virus treated lungs. These results demonstrated that AdExTek potently inhibited pulmonary tumor nodules from growing to larger

size, and indicated that the tumor growth inhibition was secondary to tumor vessel growth inhibition.

EXAMPLE III

Experimental Details

Cell lines and antibodies

Recombinant baculovirus was generated and propagated in monolayer cultured SF9 cells maintained in Grace's Insect Medium Supplemented (Gibco/BRL) at 28°C. Protein expression was carried out in suspension cultured SF9 cells in Protein-Free Insect Medium (Gibco/BRL). The R3230AC rat adenocarcinoma cell line was maintained in DMEM plus 10% fetal bovine serum (FBS, Gibco/BRL) at 37°C with 5% CO_2. Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics Inc. ECRF24, an immortalized HUVEC, was provided by Dr. Hans Pannekoek (Fontijn et al, DNA Exp. Cell. Res. 216:199-207 (1995)). Endothelial cells were maintained at 37°C, 5% CO_2 in complete endothelial cell growth medium (EGM, Clonetics, Inc) and grown on 2% gelatin (Sigma) coated plates. Endothelial cells were serum starved in endothelial cell basal medium (EBM, Clonetics, Inc). Anti-Flk antibodies (C-20 and 1158) and protein-A agarose were purchased from Santa Cruz Biotechnology. Anti phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology. Human recombinant VEGF165 was purchased from R & D Systems.

Construction of the ExFlk.6His and ExFms.6His baculovirus vectors and production of recombinant viruses

The angiogenic actions of VEGF (vascular endothelial growth factor) are mediated via two closely related endothelium-specific receptor tyrosine kinases, flk-1 and flt-1 (De Vries et al, Science 255:989-991 (1992), Terman et al, Biochem. Biophys. Res. Comm. 187:1579-1586 (1992), Shibuya, Adv. Cancer Res. 67:281-316 (1995)). The entire extracellular domain of murine flk-1 was generated by PCR from a plasmid containing a cDNA encoding the extracellular and transmembrane domains of flk-1. The forward primer consisted of nucleotides 208 to 223 with a BamHI site introduced upstream of the start codon; the reverse primer consisted of nucleotides 2493-2478 in the reverse orientation with a Cla I site introduced at the 5' end. The resulting PCR product was digested with BamHI/ClaI and ligated to the same sites of an intermediate vector (BSK Cla-/6His) to generate a cDNA encoding a fusion protein consisting of the entire extracellular domain of flk-1 with a 6 histidine tag at the C terminus (BSK/ExFlk.6His). A 2.3 kb EcoR I/Not I fragment from BSK/ExFlk.6His was subcloned into the same sites of pVL 1393, a baculoviral expression transfer vector (Pharmingen). pVL 1393/ExFlk.6His and Baculogold baculoviral DNA (Pharmingen) were co-transfected into SF9 cells for production of the recombinant baculovirus (BvExFlk.6His) according to the manufacturer's instructions. Second passage virus was used to infect serum-free SF9 cells for ExFlk.6His protein production. The same approach was used to generate a recombinant baculovirus (BvExFms.6His) expressing the entire extracellular domain of the human c-fms receptor fused to a 6-histidine tag at the C terminus.

Purification of ExFlk.6His and ExFms.6His proteins

Suspension cultured SF9 serum free insect cells (1 liter) were infected with approximately 1pfu/cell of second passage BvExFlk.6His or BvExFms.6His for 54 hours at 28°C. Cells were removed by centrifugation at 3000 rpm(Sorvall) for 20 minutes at 4°C. The supernatant was dialyzed against 8 liters of PBS pH 8.0 for 48 hours with one change of buffer. The dialyzed supernatant was then incubated with 4ml of Ni^{++}NTA resin (Qiagen). After 1 hour at room temperature, the resin-bound ExFlk.6His protein or ExFms.6His protein was loaded onto a 10ml column. The column was then washed with 200ml of wash buffer (50mM NaH_2PO_4, 300mM NaCl and 20mM imidazole pH 8.0), and the protein was eluted with elution buffer (wash buffer plus 250mM imidazole) followed by a buffer change to PBS pH 7.2 by ultra filtration (Centricon 10 from Amicon Co.). Mock control material used in the tumor study was generated from the supernatant of uninfected SF9 cells following the same purification procedure. Aliquots of purified ExFlk.6His and ExFms.6His proteins were analyzed by SDS-PAGE on a 7.5% gel.

Binding of ExFlk.6His to VEGF

Purified ExFlk.6His was radiolabelled with $Na^{125}I$ (Amersham) using a modified Iodo-Gen precedure (Zalutsky et al, Can. Res. 49:2807-2813 (1989)) to a specific activity of 2866 cpm/ng. Labeled ExFlk.6His was separated from free ^{125}I by gel filtration on a Sephadex G-25 column. There was >95% precipitability with trichloroacetic acid.

Binding of ExFlk.6His to VEGF was done basically as described by Duan et al (Duan et al, J. Biol. Chem. 266:413-418 (1991)). Human recombinant VEGF was diluted (25mM Hepes pH 7.4/75mM NaCl/20mM $NaHCO_3$) and

10ng/well of VEGF in 100µl was coated onto 96-well plates (Dynatech plates with detachable wells) overnight at 4°C. The wells were washed once with blocking buffer (25mM Hepes pH 7.4, 100mM NaCl, 0.5% gelatin, 20µg/ml BSA) and then blocked with the same buffer for 2 hours at room temperature. Increasing amounts of ^{125}I labeled ExFlk.6His (0.03-300nM) in binding buffer (25mM Hepes pH7,4, 100mM NaCl, 20µg/ml BSA, 0.5µg/ml heparin) were added to each well and incubated for 1 hour at room temperature. Nonspecific binding was determined by incubation of ^{125}I-labeled ExFlk.6His in the presence of 60-fold excess of unlabeled ExFlk.6His. The wells were washed 3 times with binding buffer and counted in a gamma counter. Scatchard analysis of binding was performed with the aid of the Scatchard Fit program.

Flk-1 activation assay

ECRF cells, a transformed HUVEC line expressing Flk-1, were grown in EGM in a 60 mm dish (Fontijn et al, DNA Exp. Cell. Res. 216:199-207 (1995)). Upon reaching confluence, the cells were serum starved in EBM overnight followed by stimulation with human recombinant VEGF at 20 ng/ml plus different amounts of ExFlk.6His at 37°C for 5 minutes. The cells were then washed with ice cold PBS 3 times on ice and then lysed with lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% triton X-100, 2 mM EDTA) supplemented with 1 mM PMSF (a protease inhibitor), 10 ng/ml leupeptin, 1 mM sodium vanadate. Flk was immunopreciptated with anti-Flk antibody (antibody 1158, Santa Cruz) for 4 hours at 4°C, and immuncomplexes were collected by addition of protein A agarose beads. After thorough washing with TBST (20 mM Tris pH 8.0, 150 mM NaCl, 0.1 % Tween 20), the bound proteins were eluted with SDS sample buffer, subjected to SDS-PAGE and transferred to nitrocellulose

filter. The filter was blocked with 5 % milk in TBST, and incubated with anti phosphotyrosine antibody (4G10) in TBST buffer for 1 hour at room temperature. With TBST washes in between each step, the filter was incubated with a HRP (horse radish peroxidase) secondary antibody conjugate and detected with ECL working solution (Amersham Corp) for 1 min, and exposed to x-ray film. The same filter was striped with TBST, pH 2.4 and reblotted with anti-Flk antibody (antibody c-20, Santa Cruz).

Endothelial cell proliferation assay

HUVECs were plated in 24 well plates at a density of 25000 cells per well. After 24 hours, the media was replaced by endothelial cell basal media (EBM as quiescence media, Clonetics, Inc) and incubated for an additional 24 hours. The old media was then replaced with either fresh quiescence media alone as a control, or quiescence media plus human recombinant VEGF at 10ng/ml, or quiescence media plus 10ng/ml VEGF and purified ExFlk.6His at 2.5μg/ml, or quiescence medium plus 10ng/ml VEGF and purified control ExFms.6His at 2.5μg/ml. The cells were incubated for 24 hours followed by a 3 hour pulse-labeling with 2μCi/ml ^{3}H-thymidine (Amersham). The reaction was stopped by aspirating the media and washing the cells with Hanks Balanced Salt Solution (Gibco/BRL). The DNA was precipitated by treating the cells with cold 10% TCA at 4°C for 30 min followed by an absolute ethanol wash. The precipitated material was resuspended in 0.5ml of 0.5M NaOH, and ^{3}H-thymidine incorporation was determined from a 400μl aliquot using Beckman LS6000SC scintillation counter.

Endothelial cell migration assay

The rate of migration of HUVECs was determined by using a modified Boyden chamber assay as described by Clyman et al (Cell Adhesion and

Commun. 1:333 (1994)). Briefly, Polycarbonate filter wells (Costar Transwell with an 8μm pore size) were coated with 2% gelatin in PBS for 30 min at room temperature and subsequently incubated at 37°C for 1 hour with DMEM containing 0.1% BSA (DMEM/BSA). Confluent HUVECs were trypsinized, pelleted by centrifugation, washed with DMEM/BSA to remove residual serum and resuspended in fresh DMEM/BSA to a final concentration of 2×10^6 cells/ml. Aliquots of cells (1×10^5) were applied to the upper chamber of the filter wells. The filter inserts with cells were placed in wells of a 24 well culture plate containing either 600μl of DMEM/BSA alone as control, or DMEM/BSA plus human recombinant VEGF at 10ng/ml, or DMEM/BSA plus VEGF at 10ng/ml pre-incubated with ExFlk.6His or pre-incubated with ExFms.6His at 2.5μg/ml for 30 min at room temperature. After a 4 hour incubation at 37°C, the cells which have migrated to the lower surface of the filter inserts were fixed with 10% formalin (Fisher) and stained with Harris' hematoxylin (Fisher). Six randomly selected high power (400X) fields were counted on each filter.

Cross-linking experiments

600ng of radio-labeled 125I-ExFlk.6His (2866cpm/ng) generated as described above was incubated at room temperature for 30 minutes with an equimolar amount of VEGF in a final volume of 500μl of binding buffer (DMEM plus 0.5μg/ml of heparin) . The binding mixture was then added to 90% confluent ECRF24 cells (60mm dish) and incubated for 4 hours at 4°C. The cells were then washed 3 times with ice cold PBS followed by cell lysis in either 0.5ml of 1M NaOH for direct counting of cell surface binding or with 1.5mM BS3 (Pierce) in PBS for cross-linking. After a 30 minute incubation at 4°C, the cross-linking reaction was stopped by the addition of 1M Tris.HCl, pH 7.4 followed by three washes with cold PBS. The cells were then lysed at 40C for 10 min in lysis buffer

containing 50mM Tris.HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.25% Na deoxycholate and protease inhibitors. The lysate was recovered after centrifugation to remove cell nuclei and either directly analyzed by 5% SDS-PAGE followed by auto radiography or immunopreciptated with an anti-Flk antibody raised against a c-tail peptide of Flk (antibody c-20, Santa Cruz) before the SDS-PAGE analysis.

Rat corneal micropocket assays

The *in vivo* angiogenic activity of ExFlk.6His was tested in the avascular cornea of F344 female rat (Harlan Labortories, Madison, WI) as described (Polverini et al, Methods Enzymol. 198:440-450 (1991)). Briefly, each sample was combined with sterile Hydron casting solution (Interferon Sciences, New Brunswick, NJ), and the solution was pipetted onto the surface of 1.5mm diameter Teflon rods (Dupont Co. Wilmington, DE). The pellets were air-dried in a laminar flow hood for 1 hour and refrigerated overnight. The following day, pellets were rehydrated with a drop of PBS and then placed in a surgically created pocket within the corneal stroma, 1.5mm from the limbus. Corneas were observed every other day until day 5 or 7 when the animals were anesthetized and perfused with lactated ringers solution followed by colloidal carbon solution to enumerate the vessels. Responses were scored as positive when vigorous and sustained directional ingrowth of capillary sprouts and hairpin loops toward the implant were detected. Negative responses were recorded when no growth was detected or when there was only an occasional sprout or hairpin loop with no evidence of sustained growth. Negative controls consisted of Hydron pellets containing media alone. Media was incorporated into pellets at a concentration of 1μg of total protein per pellet. Soluble receptor proteins were incorporated into pellets at 100ng per pellet. Histological examination of representative corneas

revealed that nonspecific inflammation was not a contributing factor in any of the corneal responses except for occasional neutrophils found in the limbus of both control and test corneas.

Tumor window chamber model

R3230AC tumors were grown in cutaneous window chambers in Fischer 344 rats (age 12-14 weeks, weight 140-160 grams, purchased from Charles River Labs, Raleigh, NC.) as previously described (Papenfuss et al, Microvascu. Res. 18:311-318 (1979)). Briefly, two 1cm diameter holes were dissected in opposing epithelial surfaces of the dorsal skin flap as it was retracted away from the posterior surface of the back. The underlying tissue was dissected away until two fascial planes with associated vasculature remained. 100µl of ExFlk.6His protein (0.5µg/µl) or 100µl of mock purified control solution was injected between the fascial planes. A 0.1mm^3 piece of tumor from a donor rat was then placed onto the fascial plane and an additional 100µl of protein or control solution was added and the chambers were sealed with glass cover slips. A pair of tumor window chambers were done at each time, one treated with ExFlk.6His and the other with control solution. The tumor implants in each pair were taken from the same region of grossly viable donor tumor tissue. Ten days after implantation, tumors in window chambers (200µm thick) were photographed using transillumination and a dissecting microscope (Zeiss, Stemi SV6) for vascular length density measurement and were subsequently harvested for H & E staining.

Measurement of tumor volume and tumor vascular length density

To obtain an estimate of tumor volume, H & E stained sections representing the largest cross-sectional area of each tumor were photographed and the thickness (t) and the diameter (d) of tumors were measured from the

photographs. Tumor volumes, which were assumed to approximate a flat cylinder in shape, were calculated using the formula:

$$\text{Tumor volume } (mm^3) = 3.14t(d/2)^2$$

Tumor vascular length density as an indicator of tumor vascularity was measured from photographs of 10 day old tumors within the window chamber using a previously described method (Dewhirst et al, Radiat. Res. 130:345-354 (1992)). 3-5 areas inside the tumor were randomly selected for measurement. The vascular length density in mm/mm^3 was calculated using the formula:

$$\text{Length density} = N/(4gdt)$$

where N is the average number of intersections between vessels and grid per sheet; g is number of blocks in grid (=54); d is length of one grid square calibrated by a micrometer image at the same magnification (=0.1333mm); and t is measured depth of field through which microvessels could be discerned (=0.2mm).

Tumor cell toxicity assay

R3230AC tumor cells were seeded at $2X10^4$/well into 24-well plates and maintained in the presence of purified ExFlk.6His protein (3μM) or control solution. Cell morphology was monitored each day by light microscopy. Cells were trypsinized, suspended in PBS containing 0.02% trypan blue (Gibco/BRL) and live cells were counted with a hemacytometer on each following day for three days.

Statistics

Results are reported as mean±SE for tumor volume and tumor vascular length density for each group. A two-tailed Student's t test was used to analyze

statistical differences between control and ExFlk.6His treated groups. Differences were considered statistically significant at $p<0.05$.

Results

Design and Production of A Recombinant Soluble VEGF Receptor (ExFlk.6His)

To generate a soluble VEGF receptor, a baculovirus vector (BvExFlk.6His) was generated directing the expression of a fusion protein consisting of the entire extracellular domain of the murine VEGF receptor, flk-1, fused to a 6 histidine-tag at the C-terminus (Figure 8A). ExFlk.6His protein was purified from the supernatant of SF9 cells infected with BvExFlk.6His virus by one-step Ni^{++} NTA resin chromatography. As controls, ExFms.6His was purified from the supernatant of SF9 cells infected with a baculovirus vector directing the expression of a fusion between the extracellular domain of the CSF-1 receptor, c-fms, fused to a 6 histidine-tag. A mock control was purified from uninfected SF9 cells using the same protocol. By this approach, ExFlk.6His and ExFms.6His proteins were purified to near homogeneity, yielding a single major band with the expected molecular masses of approximately 105kD and 70kD, respectively (Figure 8B).

ExFlk.6His protein binds VEGF, blocks the activation of endothelial VEGF receptor and neutralizes VEGF stimulated endothelial proliferation and migration in vitro

To determine the binding affinity of ExFlk.6His with VEGF, binding assays were done by adding ^{125}I labeled ExFlk.6His to microtiter wells pre-coated with human recombinant VEGF. Bound ^{125}I ExFlk.6His was then detected by counting the washed wells in a Gamma counter. Under the conditions

of this assay, ExFlk.6His demonstrated high affinity (Kd=16nM) saturable VEGF binding (Figure 9A). This binding was specifically competed by addition of cold ExFlk.6His and no competition of binding was observed with excess cold ExFms.6His.

The ability of ExFlk.6His to block activation of VEGF receptors on cultured endothelial cells was tested. Stimulation of endothelial cells (ECRF) with VEGF produced easily detectable autophosphorylation of endogenous Flk-1. This high level phosphorylation of Flk-1 was significantly blocked by addition of ExFlk.6His in a concentration dependent manner. Even in the presence of a saturating concentration of VEGF, a 3 fold molar excess of ExFlk.6His resulted in substantial inhibition of Flk-1 activation. With the addition of a 12 fold molar excess of ExFlk.6His, the receptor phosphorylation level was reduced almost to baseline. This result clearly demonstrated the ability of ExFlk.6His to block activation of endogenous VEGF receptors.

Next, the ability of ExFlk.6His to block VEGF stimulated mitogenesis and migration of cultured human umbilical vein endothelial cells (HUVECs) was tested. Stimulation of HUVECs with VEGF (10ng/ml) produced a characteristic 2-3 fold increase in ^{3}H-thymidine incorporation 24 hours post-stimulation ($p<0.005$) compared to un-stimulated cells (Figure 9B). This VEGF-stimulated mitogenic activity was completely blocked by simultaneous addition of ExFlk.6His protein (2.5μg/ml) but was not blocked by ExFms.6His at the same concentration ($p<0.0005$). Similarly, HUVEC migration rate in a modified Boyden chamber assay increased approximately 3 fold by the addition of VEGF at 10ng/ml ($p<0.0005$), and this increase in migration was reduced to background levels after pre-incubation of VEGF with ExFlk.6His (2.5μg/ml) but not with ExFms.6His at the same concentration ($p<0.0005$, Fig. 9C). These data

demonstrate that ExFlk.6His could specifically bind VEGF and inhibit VEGF-mediated endothelial cell responses.

ExFlk.6His forms a VEGF-dependent heterodimer with endogenous VEGF receptors on the surface of cultured endothelial cells

To gain insight into the mechanism of ExFlk.6His inhibition of VEGF mediated endothelial responses, ^{125}I-ExFlk.6His was mixed with VEGF to form a ^{125}I-ExFlk.6His/VEGF complex. This pre-formed complex was then tested for its ability to bind to VEGF receptors on the surface of cultured endothelial cells (Figure 10A). In these experiments, the binding of ^{125}I-ExFlk.6His to the endothelial cell surface could only be detected in the presence of VEGF; no ^{125}I-ExFlk.6His binding was detected in the absence of VEGF or in the presence of FGF. Excess of unlabeled ExFlk.6His (30X) totally competed off this binding. When ^{125}I-ExFlk.6His was covalently cross-linked to endothelial cells in the presence of VEGF, a large molecular mass complex was revealed (Figure 10B, lane 2). No complex formation was detected in the absence of VEGF (Figure 10B, lane 1), or in the presence of FGF (Figure 10B, lane 4), and excess, unlabeled ExFlk.6His competitively blocked complex formation (Figure 10B, lane 3). These data are consistent with the formation of a VEGF mediated heterodimer between ^{125}I-ExFlk.6His and endogenous VEGF receptors. To further confirm the complex indeed contained the cell surface receptor, the cross-linked complex was immunopreciptated with an antibody against the c-tail of Flk-1 (antibody 1158, Santa Cruz) and analyzed by SDS-PAGE and autoradiography. Again, consistent with the formation of VEGF mediated receptor heterodimer, a single high molecular complex containing both the endogenous cell surface receptor and the radiolabeled soluble receptor was immunopreciptated only in the

presence of VEGF. The formation of such a heterodimeric complex between ExFlk.6His and cell surface VEGF receptors indicates that ExFlk.6His could function as a "dominant negative" inhibitor of VEGF receptor activation and should be a potent inhibitor of angiogenesis *in vivo*.

ExFlk.6His inhibits corneal angiogenesis in vivo

To determine whether ExFlk.6His could inhibit angiogenesis in vivo, a rat corneal micropocket assay was used (Polverini et al, Methods Enzymol. 198:440-450 (1991)). When pellets containing R3230AC tumor cell conditioned media were implanted into rat corneas, a strong angiogenic response was seen as early as 5 days after implantation in comparison with Hydron pellets containing fresh media when no angiogenic response was seen (Table 2). With the addition of ExFlk.6His (100ng) to the pellets containing tumor cell conditioned media, the angiogenic response induced by tumor conditioned media was totally blocked in 5 corneas (72%) and a weak growth was seen in 2 corneas (28%). Addition of a control protein, ExFms.6His, to pellets containing tumor cell conditioned media did not block vessel formation. These results demonstrated that ExFlk.6His could inhibit angiogenesis induced by tumor conditioned media in vivo and suggested its potential application as an anti-angiogenic agent for cancer therapy.

Table 2. Inhibitory Effect of ExFlk.6His on Angiogenic Response Induced by Tumor Conditioned Media

Samples	Proportion of Corneal Angiogenic Responses (%)		
	Negative	Weakly Positive	Strong Positive
Fresh Media	3/3 (100%)*		
Conditioned Medium (CM)			4/4 (100%)
ExFlk.6His	2/2 (100%)		
Ex.Fms.6His	4/4 (100%)		
CM + ExFlk.6His	5/7 (72%)	2/7 (28%)	
CM + ExFms.6His			3/3 (100%)

*n/m (x):m is the number of rats used in each experiment; n the number of rats with the indicated response; x is the percentage of rats with the indicates response

ExFlk.6His inhibits tumor growth in cutaneous "window chambers"

To evaluate whether the VEGF neutralizing protein ExFlk.6His could reduce tumor growth and tumor angiogenesis in vivo, a rat cutaneous window chamber model bearing an R3230AC mammary tumor was used (Dewhirst et al, Radiat. Res. 130:345-354 (1992)). Previous work has demonstrated that small fragments of tumor ($0.1mm^3$) placed in a window chamber become vascularized and undergo rapid growth within 10-14 days (Dewhirst et al, Radiat. Res. 130:345-354 (1992)). Typically, vascularization of tumors in the window chamber is first detected at about 5 days after implantation and is followed by a rapid growth phase of the tumor. The rapid growth and vascularization of tumor grown in the tumor window chamber together with the small volume of the tumor window made it possible to test the ability of ExFlk.6His to block tumor angiogenesis using a single, small dose of protein.

In this model, a single dose of purified ExFlk.6His protein (100μg) or mock control solution was administered directly into the window chamber at the time of tumor implantation. After 10 days, gross inspection of live tumors in the tumor window and examination of histological sections demonstrated that tumors (n=10 pairs) treated with ExFlk.6His protein were thinner, smaller, contained more connective tissue, and had a less well developed vasculature compared to tumors in the control group. Comparison of tumor volumes measured from the histological sections confirmed that the ExFlk.6His treated tumors were on average 75% smaller than the control tumors (n=10 pairs, $p<0.005$). There was no obvious gross difference in the inflammatory response in control vs ExFlk.6His treated windows. These results showed that blockade of the VEGF pathway by ExFlk.6His could inhibit tumor growth and suggested that ExFlk.6His blocked tumor growth by inhibiting tumor angiogenesis.

ExFlk.6His inhibits tumor vascularization

To determine whether inhibition of tumor growth by ExFlk.6His was secondary to inhibition of tumor angiogenesis, tumor vascular length density was measured from photomicrographs of live tumor window chambers bearing ExFlk.6His treated or control treated tumors 10 days after implantation. Consistent with the sparse appearance of the vasculature of ExFlk.6His treated tumors, there was an approximately 50% reduction in tumor vascular length density in ExFlk.6His treated tumors vs. control treated tumors (n=10 pairs, $p<0.005$). This finding is in accordance with the ability of ExFlk.6His protein to neutralize VEGF mediated endothelial responses in vitro, and to block tumor cell conditioned media stimulated angiogenesis in the rabbit cornea. It is also consistent with the notion that the primary action of the ExFlk.6His protein is to inhibit tumor neovascularization.

ExFlk.6His does not directly affect tumor cell proliferation or viability

To further confirm that the primary effect of ExFlk.6His protein was on the tumor vasculature, the cytotoxicity of the ExFlk.6His protein on cultured R3230AC cells was assayed. When cells were cultured in the presence of 3μM ExFlk.6His protein, which was roughly equivalent to the protein concentration used in tumor window chambers, no significant difference in tumor cell proliferation or viability was observed compared to control Fig. 11. Thus, inhibition of tumor growth by ExFlk.6His protein was not mediated by direct tumor toxicity further indicating that the primary action of ExFlk.6His is to inhibit tumor angiogenesis.

* * *

All documents cited above are hereby incorporated in their entirety by reference.

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. A fusion protein comprising an extracellular domain of an endothelium specific receptor, free of transmembrane domain sequences of said endothelium specific receptor, and a non-endothelium specific receptor sequence that does not mediate formation of dimers or oligomers or is non-immunogenic, wherein said non endothelium specific receptor sequence is not Fc.

2. The protein according to claim 1 wherein said extracellular domain comprises a Tie 2 extracellular domain.

3. The protein according to claim 2 wherein said extracellular domain comprises a human Tie 2 extracellular domain.

4. The protein according to claim 1 wherein said non-endothelium specific receptor sequence comprises polyhistidine.

5. The protein according to claim 1 wherein said non-endothelium specific receptor sequence consists essentially of 6 histidine residues.

6. The protein according to claim 1 wherein said non-endothelium specific receptor sequence is present in said fusion protein C-terminal to said extracellular domain.

7. A pharmaceutical composition comprising the fusion protein according to claim 1 and a pharmaceutically acceptable carrier.

8. A nucleic acid encoding the fusion protein according to claim 1.

9. The nucleic acid according to claim 8 wherein said extracellular domain is a Tie 2 extracellular domain.

10. A recombinant molecule comprising the nucleic acid according to claim 8 and a vector.

11. The molecule according to claim 10 wherein said vector is a viral vector.

12. The molecule according to claim 11 wherein said viral vector is an adenoviral vector.

13. The molecule according to claim 10 wherein said nucleic acid is present in said vector operably linked to a promoter.

14. A host cell comprising the molecule according to claim 10.

15. A method of producing a fusion protein comprising culturing the host cell according to claim 14 under conditions such that said nucleic acid is expressed and said fusion protein is thereby produced.

16. A method of inhibiting angiogenesis at a body site of a patient comprising contacting said body site with an amount of said protein according to claim 1 sufficient to effect said inhibition.

17. The method according to claim 16 wherein the body site is a tumor site.

18. The method according to claim 16 wherein an expression construct comprising a nucleic acid encoding said fusion protein is administered to said patient under conditions such that said nucleic acid is expressed and said fusion protein is thereby produced and contacted with said body site.

19. A method of inhibiting growth of a tumor in a patient comprising contacting said tumor with an amount of said protein according to claim 1 sufficient to inhibit angiogenesis and thereby inhibit said growth.

20. A method of screening a test compound for its ability to bind an endothelium specific receptor extracellular domain comprising contacting said test compound with said fusion protein according to claim 1 and determining whether binding of said test compound to said fusion protein occurs.

21. The method according to claim 20 wherein said fusion protein is bound to a solid support via said non endothelium specific receptor sequence.

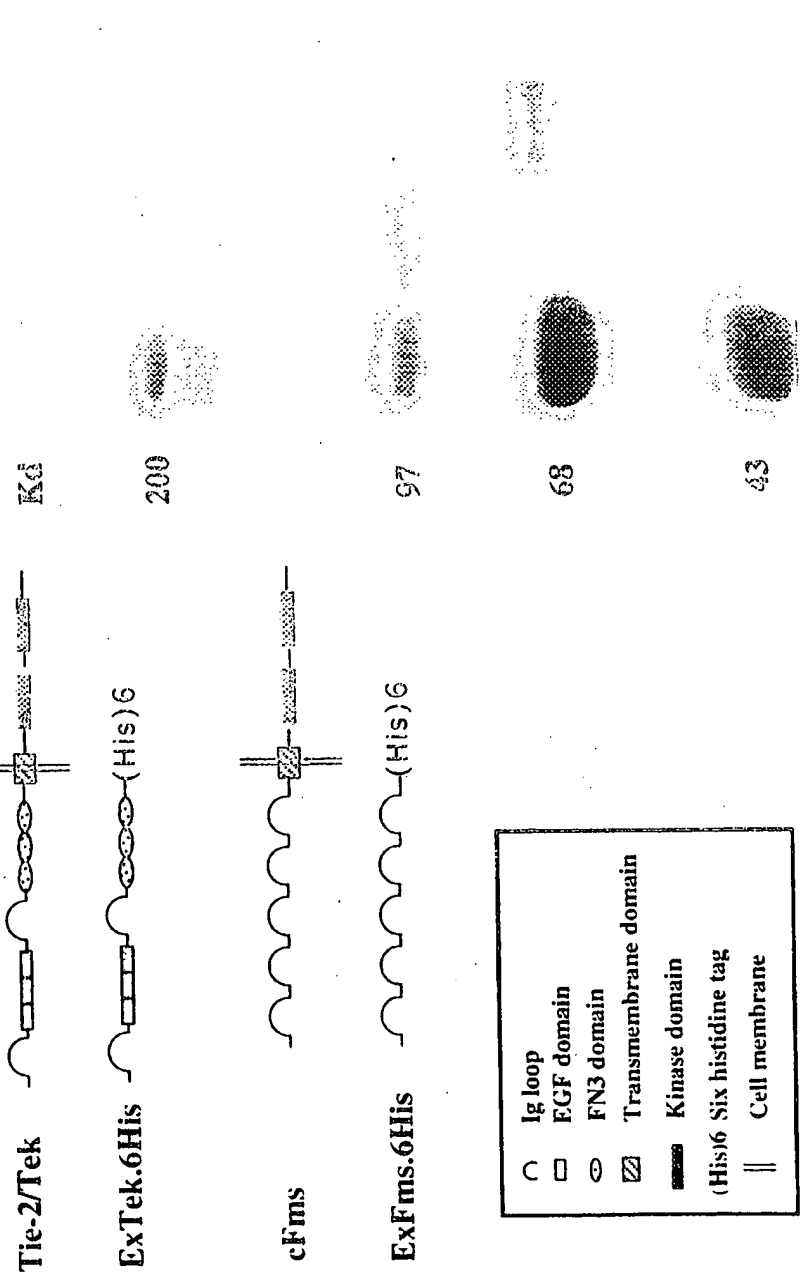
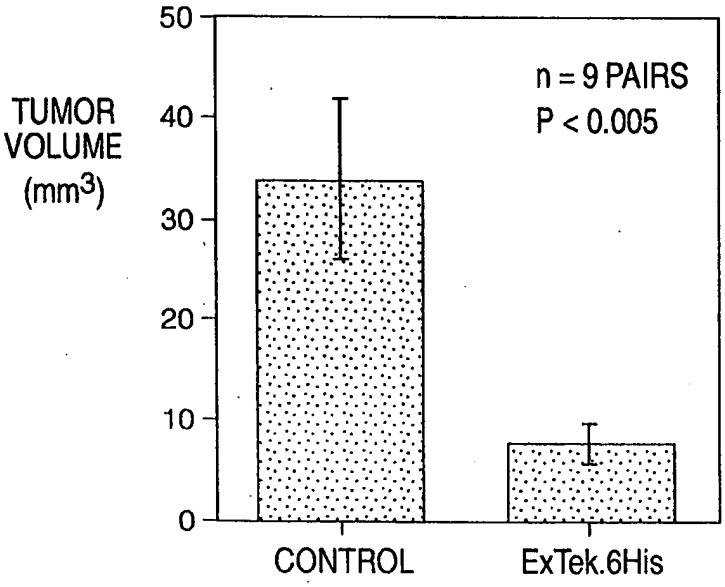

Tie-2/Tek
ExTek.6His
cFms
ExFms.6His
(His)6
Ig loop
EGF domain
FN3 domain
Transmembrane domain
Kinase domain
(His)6 Six histidine tag
Cell membrane

Fig. 1A

1
2
3
4
Kd
200
97
68
43

Fig. 1B

Fig. 2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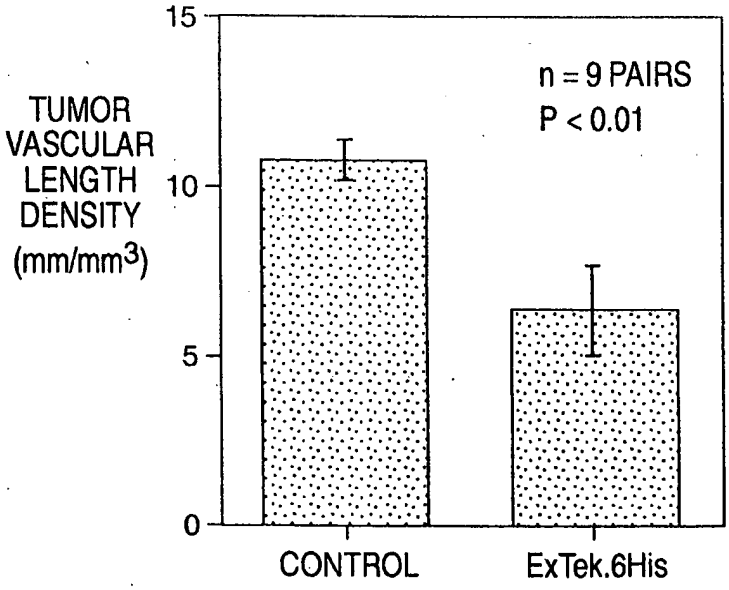
40
30
20
10
0
n = 9 PAIRS
P < 0.005
CONTROL
ExTek.6His

Fig. 3


TUMOR VASCULAR LENGTH DENSITY (mm/mm3)
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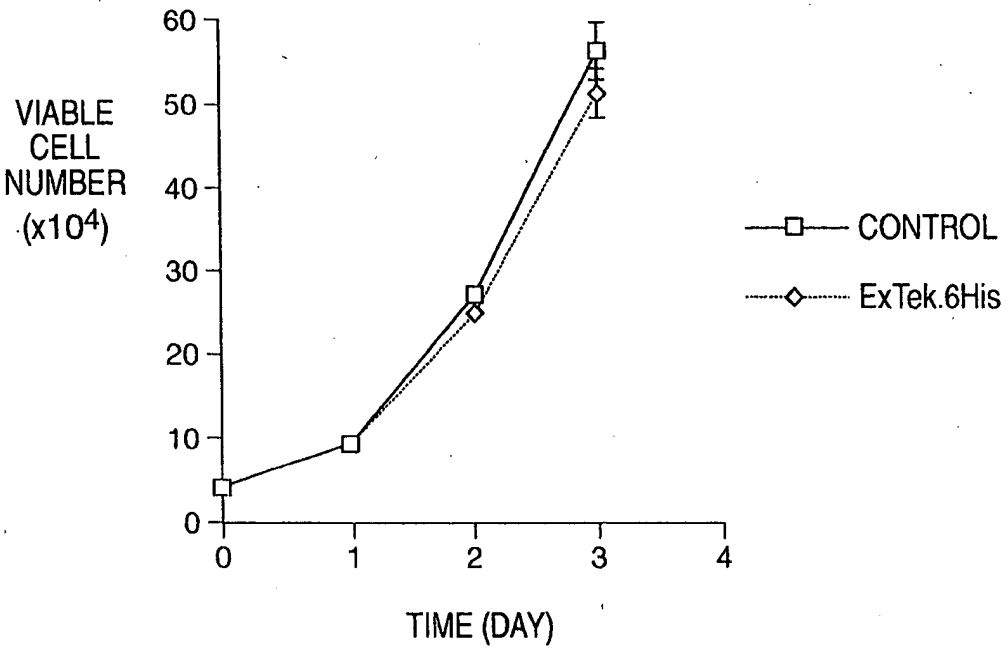
10
5
0
n = 9 PAIRS
P < 0.01
CONTROL
ExTek.6His

Fig. 4


VIABLE CELL NUMBER (x10⁴)
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40
30
20
10
0
0
1
2
3
4
TIME (DAY)
CONTROL
ExTek.6His

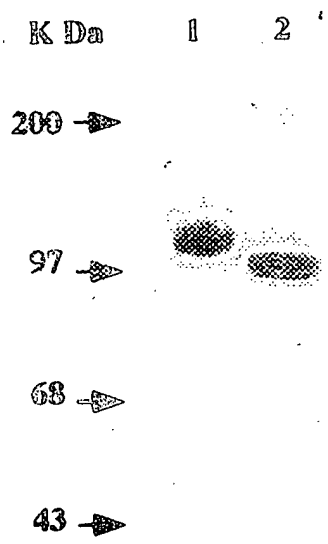

K Da
1
2
200
97
68
43

Fig. 5A

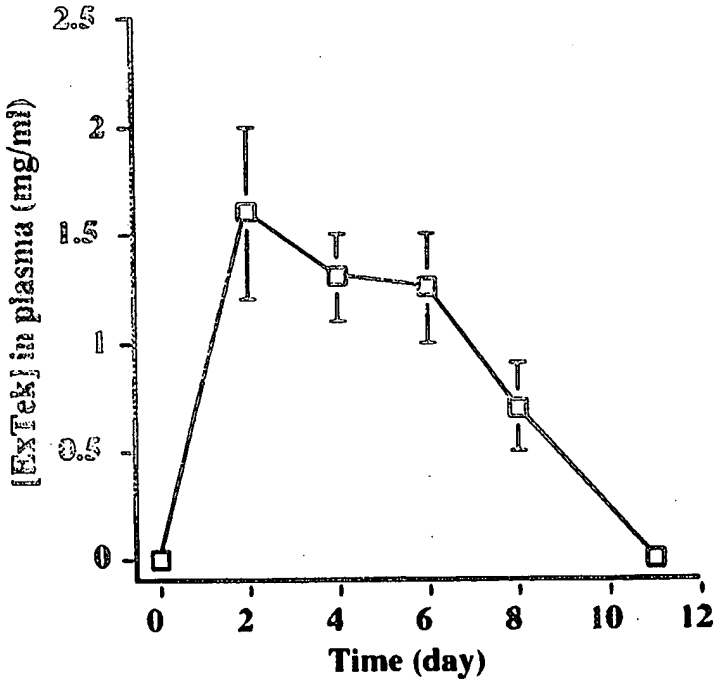
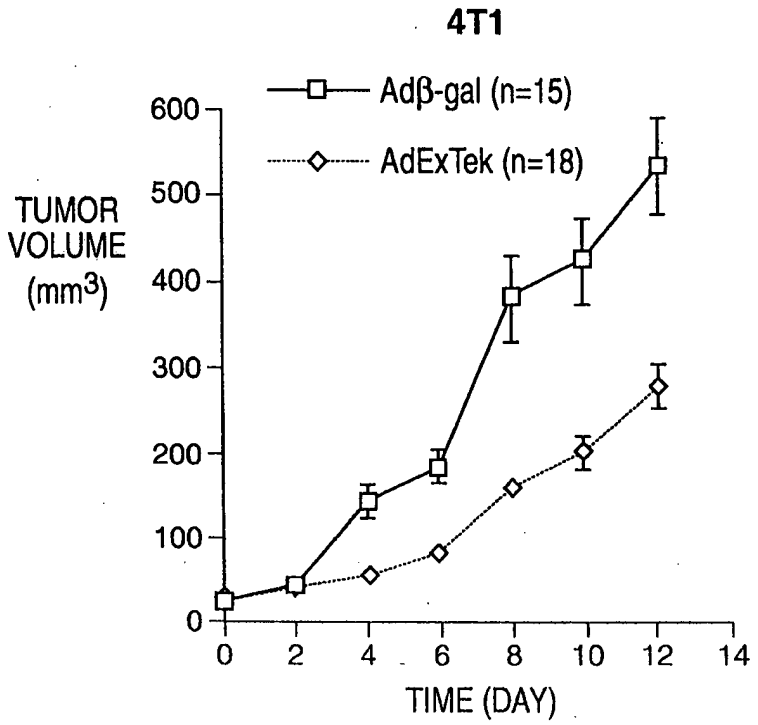

[ExTek] in plasma (mg/ml)
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
Time (day)

Fig. 5B


4T1
Adβ-gal (n=15)
AdExTek (n=18)
TUMOR VOLUME (mm3)
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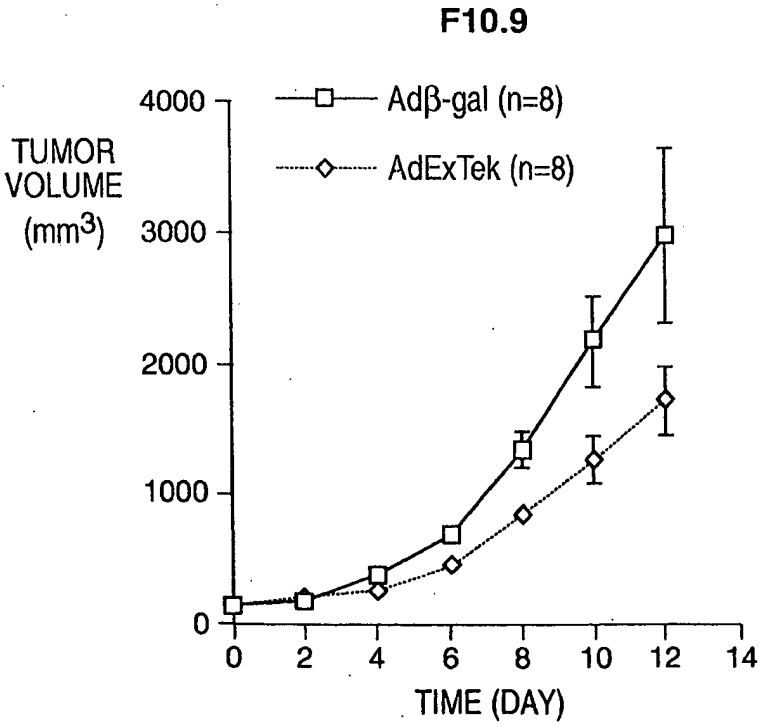
500
400
300
200
100
0
0
2
4
6
8
10
12
14
TIME (DAY)

Fig. 6A


F10.9
Adβ-gal (n=8)
AdExTek (n=8)
TUMOR VOLUME (mm3)
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3000
2000
1000
0
0
2
4
6
8
10
12
14
TIME (DAY)

Fig. 6B

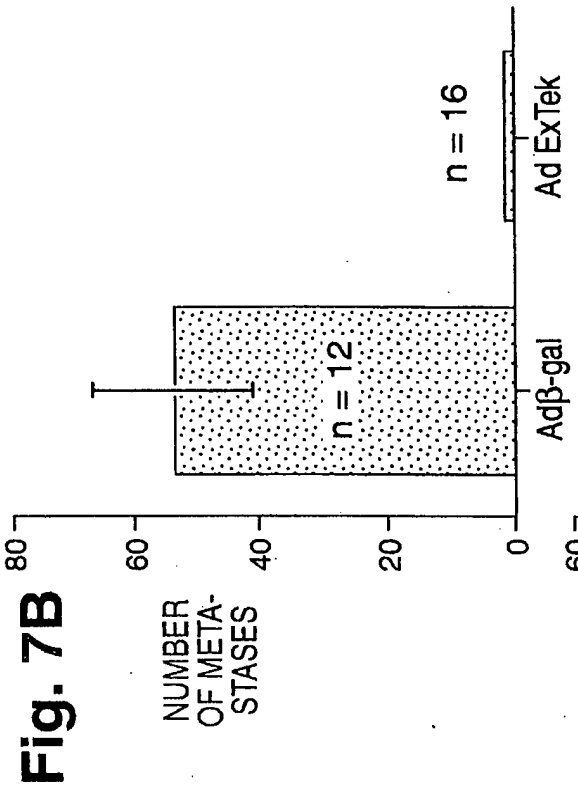


Fig. 7B
NUMBER OF META-STASES
0
20
40
60
80
n = 12
n = 16
Adβ-gal
Ad ExTek

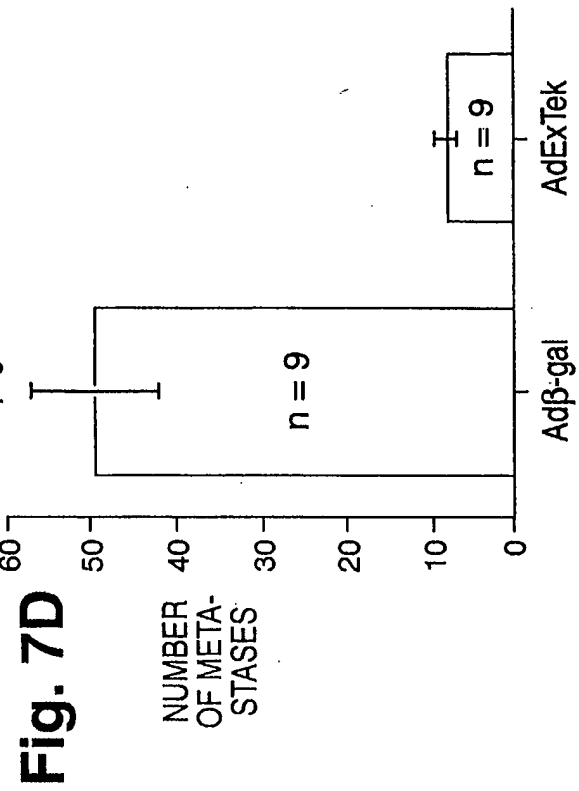


Fig. 7D
NUMBER OF META-STASES
0
10
20
30
40
50
60
n = 9
n = 9
Adβ-gal
AdExTek

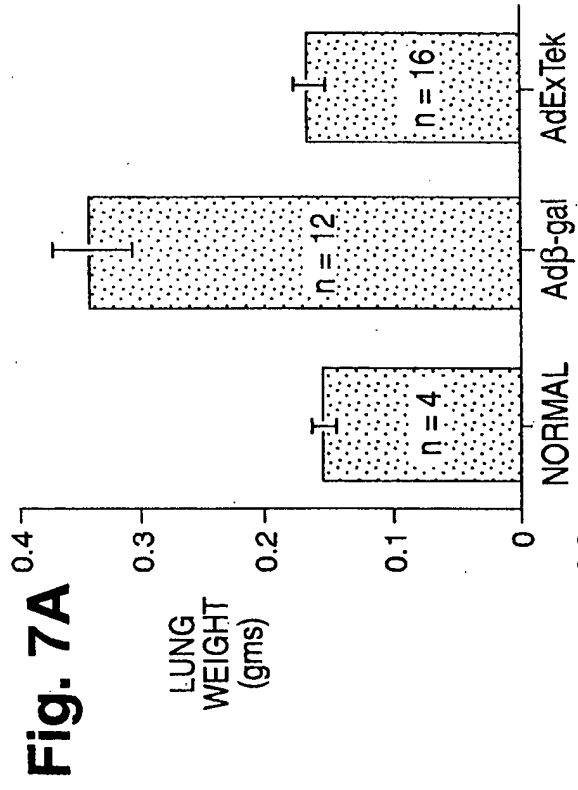


Fig. 7A
LUNG WEIGHT (gms)
0
0.1
0.2
0.3
0.4
n = 4
n = 12
n = 16
NORMAL
Adβ-gal
AdExTek

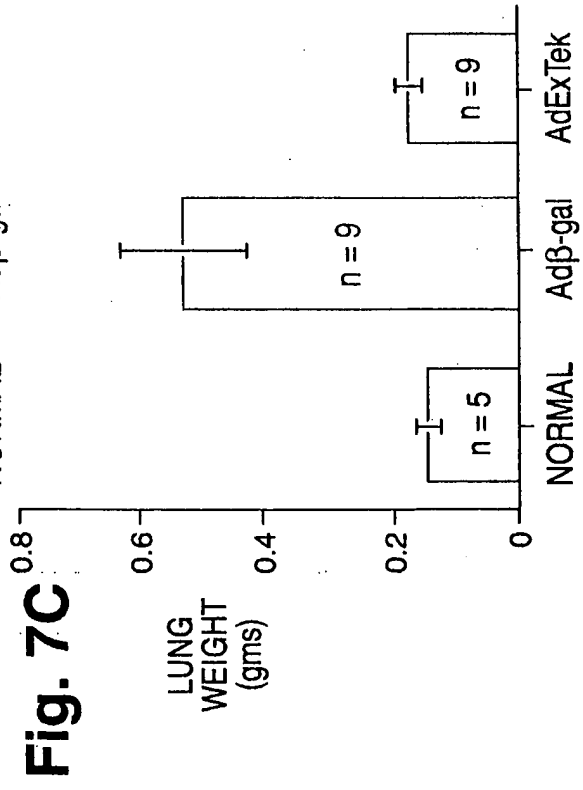


Fig. 7C
LUNG WEIGHT (gms)
0
0.2
0.4
0.6
0.8
n = 5
n = 9
n = 9
NORMAL
Adβ-gal
AdExTek

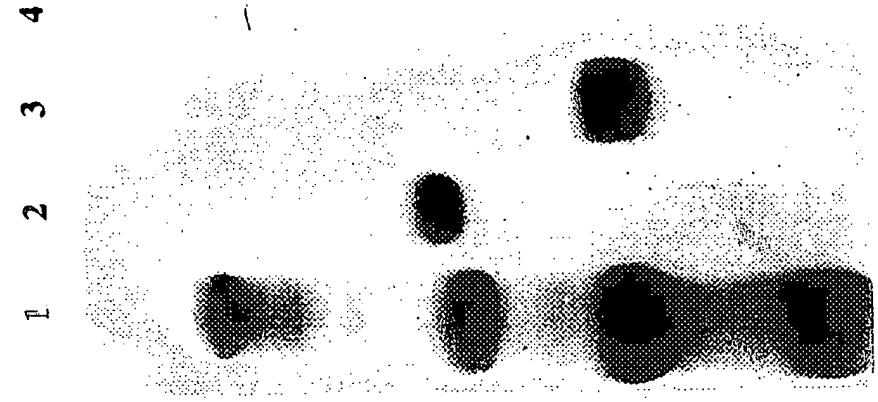

1
2
3
4
KD
200
97
68
43

Fig. 8B


Flk-1
ExFlk.6His
cFms
ExFms.6His
(His)6
Ig loop
Transmembrane domain
Kinase domain
6 Histidine tag
Cell membrane

Fig. 8A

Fig. 9A

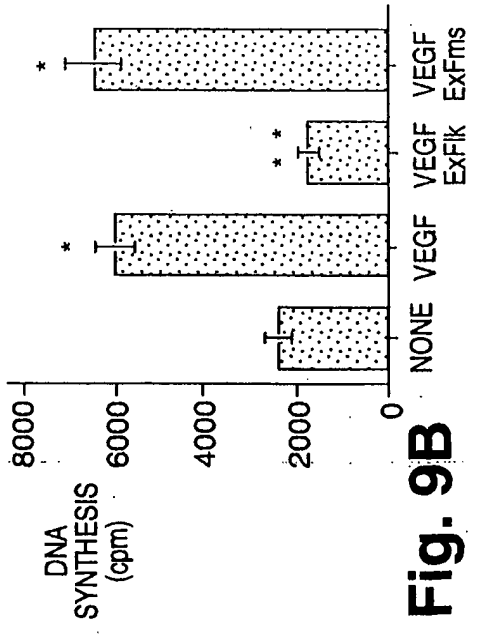


Fig. 9B

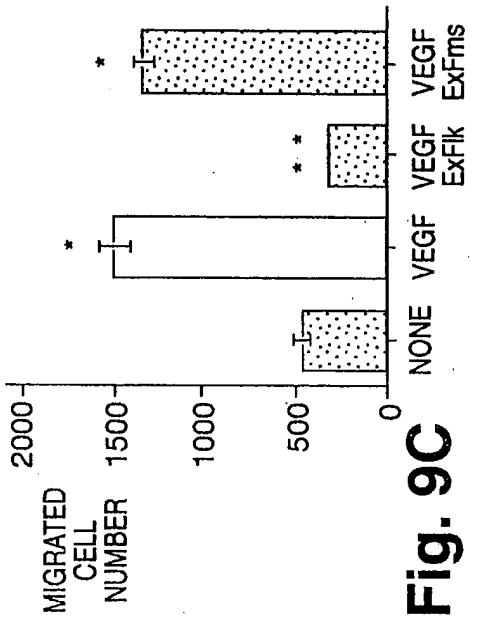


Fig. 9C

Fig. 10A

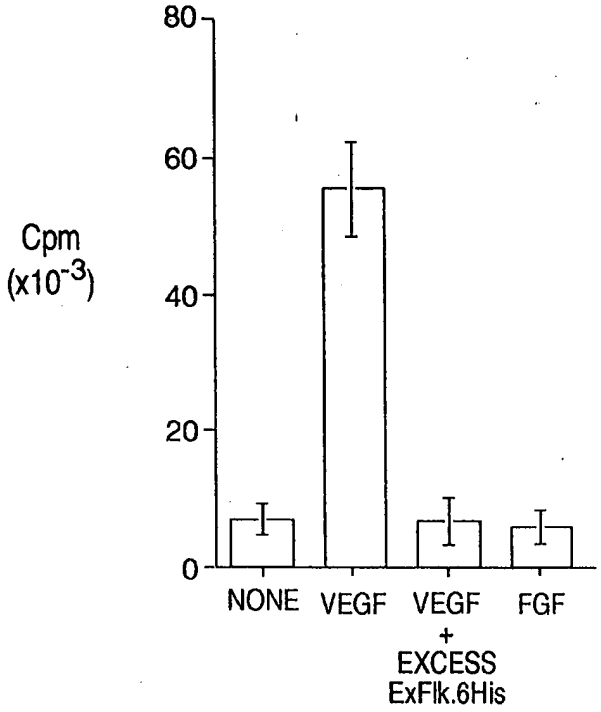


Fig. 11

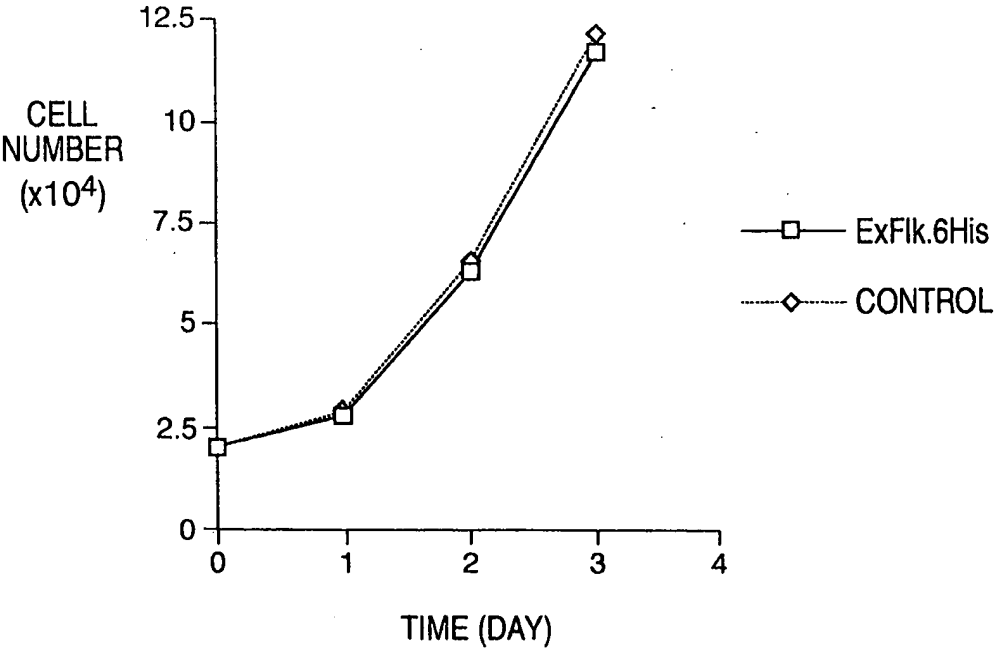
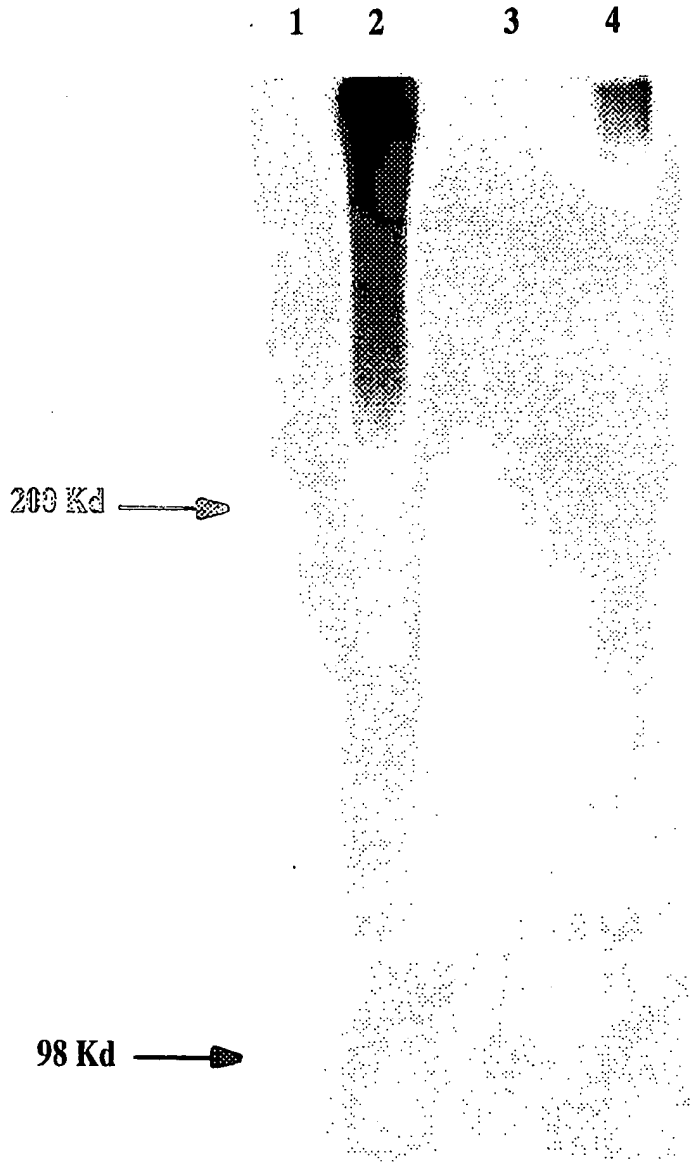


Fig. 10B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19597

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 435/7.1, 7.2, 7.8, 69.7, 71.1, 71.2, 172.3, 325, 252.3, 3201; 514/2, 8, 12, 44; 536/23.1, 23.4, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PARTANEN et al. A Novel Endothelial Cell Surface Receptor Tyrosine Kinase with Extracellular Epidermal Growth Factor Homology Domains. Molecular and Cellular Biology. April 1992, Vol.12, No.4, pages 1698-1707, especially pages 1700-1701.	1-3, 6-15
Y	US 5,338,669 A (GILLIES) 16 August 1994, column 1, lines 10-35.	1-3, 6-15
Y	US 5,488,032 A (DOWER ET AL) 30 January 1996, column 7, lines 45-65.	4-5
A	US 5,405,941 A (JOHNSON) 11 April 1995, see entire document.	1-21
A	US 5,447,860 A (ZIEGLER) 05 September 1995, see entire document.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

31 DECEMBER 1997

Date of mailing of the international search report

10 FEB 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

PREMA MERTZ

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19597

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,447,860 A (S. F. ZIEGLER) 05 September 1995 (05/09/95), see entire document.	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19597

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 15/09, 15/12, 15/63; G01N 33/53; A61K 38/16, 38/17, 38/18, 48/00; C07K 14/705, 14/71, 14/715

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/350; 435/7.1, 7.2, 7.8, 69.7, 71.1, 71.2, 172.3, 325, 252.3, 3201; 514/2, 8, 12, 44; 536/23.1, 23.4, 23.5